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Studies on Growth Inhibition of Hiochi-bacteria, Specific Saprophytes of Sake*

Part VI. Muta-aspergillic Acid as a New Growth Inhibibitant against Hiochi-bacteria

By Seiji NAKAMURA and Teruo SHIRO

A new growth inhibitant against hiochi-bacteria, $C_{11}H_{18}N_2O$, m.p. 173~4°C, named muta-aspergillic acid, has been isolated from the crystals of crude hydroxyaspergillic acid, obtained from culture filtrate of *Asp. oryzae*. Successful separation of these two compounds from each other was accomplished by counter current distribution method. The physical and chemical properties of muta-aspergillic acid as well as its physiological properties are described.

INTRODUCTION

In previous paper[1], the authors reported the isolation of an antibiotic from the culture filtrate of *Asp. oryzae*, as pale yellow crystals of m.p. 152~3°C, responsible for the active substances against hiochi-bacteria, and confirmed that this antibiotic is identical with hydroxyaspergillic acid $C_{12}H_{20}N_2O_3$. Because the authors' sample showed slightly different melting point from that of hydroxyaspergillic acid described in literatures[2,3], the authors had some doubts on the purity of the crystals of hydroxyaspergillic acid. In order to investigate the purity of authors' crystalline hydroxyaspergillic acid, m.p. 152~3°C, it was subjected to counter current distribution between ethyl acetate and M/10 citrate buffer solution (pH 6.2) and the authors succeeded in isolating a new growth inhibitant against hiochi-bacteria, $C_{11}H_{18}N_2O_3$ (m.p. 173~4°C) which was named muta-aspergillic acid. The present paper deals with isolation and some properties of this new antibiotic.

EXPERIMENTAL METHODS AND RESULTS

I) Isolation of a New Growth Inhibitant Against Hiochi-bacteria.

Asp. oryzae A-O-4 was shake-cultured and the culture medium showed an antibiotic activity against hiochi-bacteria H-1 and H-34 in the same manner as reported in the previous paper[1]. From the culture filtrate, an active crystalline substance against hiochi-bacteria, was obtained through the process shown in Table I. Although, it was proved that the properties of the antibiotic crystals were almost identical with those of hydroxyaspergillic acid, the authors had some doubts on their purity. Therefore, the authors applied the counter current distribution method for the antibiotic crystals as follows.

Fifty milligrams of the crystals were dissolved in 30 ml of ethyl acetate and subjected to 85 plates counter current distribution using ethyl acetate and M/10 Sörensen's citrate buffer solution (pH 6.2) as a solvent system. The amount of the antibiotic in each tube was determined by the spectrophotometric

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1) S. Nakamura and T. Shiro, Bull. Agr. Chem. Soc. of Japan, **23**, 65 (1959).

2) A. E. O. Menzel, O. Wintersteiner and G. Rake, *J. Bact*, **46**, 109 (1943).

3) J. D. Dutcher, *J. Biol. Chem.*, **232**, 785 (1958).

TABLE I. PROCESS OF ISOLATION OF A CRYSTALLINE ANTIBIOTIC FROM FILTRATE OF *Asp. oryzae* A-O-4

Cultured Filtrate (pH7)
- concentrated by flash evaporator
- acidified with HCl (pH 3.5)
- extracted with ether for 40 hours

Aqueous layer / Solvent layer
- concentrated in vacuo

Crude Substance
- dissolved in ethyl acetate
- extracted with 2% $NaHCO_3$ aq.

Solvent layer / Aqueous layer
- acidified with HCl (pH 3.5)
- extracted with chloroform

Aqueous layer / Solvent layer
- evaporated off chloroform

Crude crystal
- recrystallized from 50% ethyl alcohol or *N*-hexane

Pale yellow crystal (mp. 152~3°C)
yield : 500~1000mg/1 of culture filtrate

method based on the ultraviolet absorption curve for hydroxyaspergillic acid (Maximum at 337 mμ in this buffer solution). As shown in Fig. 1, the counter current distribution curve of this preparation showed two peaks. From this fact, the authors concluded that the crystals of m.p. 152~3°C were not a homogeneous compound and contained a small amount of other component. In this solvent system, the phosphate buffer solution could not be used in place of citrate buffer, because hydroxyaspergillic acid was easily decomposed to red syrupy material by the presence of phosphoric acid.

For isolation of muta-aspergillic acid, 4 g of the crude antibiotic crystals (m.p. 148°C) were dissolved in 120 ml of M/10 Sörensen's citrate buffer solution (pH 6.0) and the first counter current distribution of 90 plates was carried out using a solvent system of ethyl acetate and M/10 Sörensen's citrate buffer (pH 6.0) in Craig's apparatus of 60 ml volume (Fig. 2 (a)). Muta-aspergillic acid was distributed in tubes between 3 and 21. These fractions were combined, and the lower phase was adjusted to pH 4.2, and extracted with ether continuously. Ether solution thus obtained, was evaporated in vacuo. Also, after drying with sodium sulfate, ethyl acetate solution of the upper phase was evaporated in vacuo.

From these two phases, 50 mg of crystals, m.p. 158°C were obtained which contained relatively large amount of muta-aspergillic acid. One hundred milligrams of this crystal were dissolved in 30 ml of M/10 Sörensen's citrate buffer solution (pH 6.0). The second counter current distribution of 120 plates was carried out in Craig's apparatus using the same solvent system as in the first counter current distribution. The results are shown in Fig. 2 (b). Pure muta-aspergillic acid was distributed between the 10th and the 30th tubes. From these tubes, about 70 mg of pale yellow crystal of new antibiotic were obtained in the same way as in the first experiment. Recrystallization from hot *n*-hexane and hot aqueous ethyl alcohol gave pale yellow crystals of m.p. 173~4°C. The homogeneity of this antibiotic was determined by counter current distribution in the same way. As shown in Fig. 3, the distribution curve showed no detectable deviation from a theoretical curve. The melting point of pure hydroxyaspergillic acid obtained by the counter current distribution, m.p.

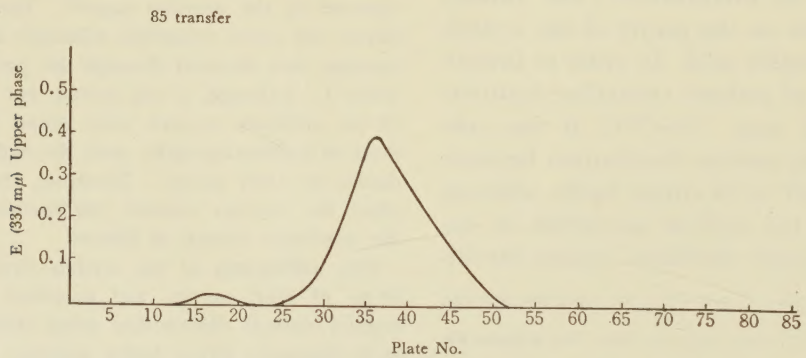

FIG. 1. Countercurrent Distribution Curve of Hydroxyaspergillic Acid (m.p. 152~3°C).
System : Ethylacetate, M/10 Citrate buffer solution (pH 6.2)

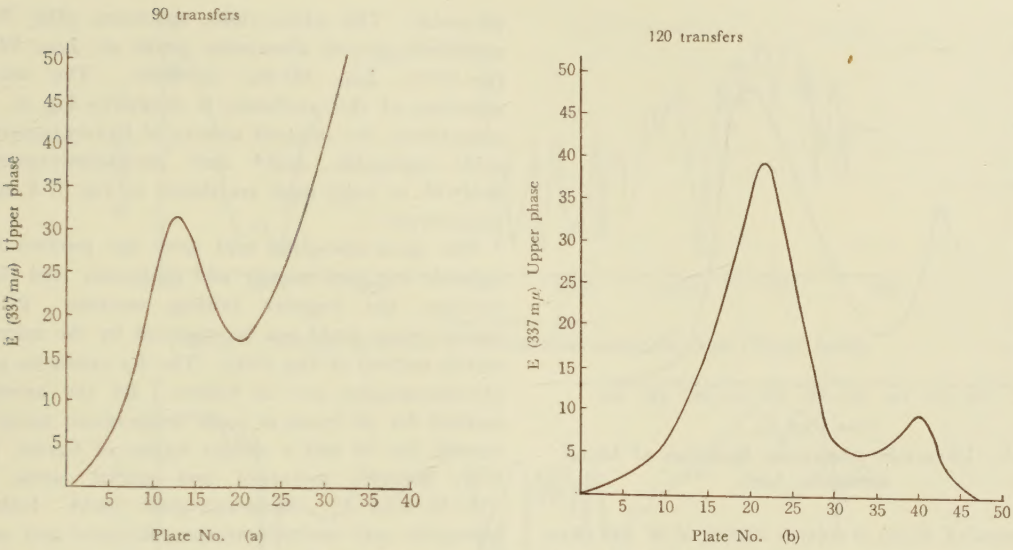

FIG. 2. Separation of Muta-aspergillic Acid and Hydroxyaspergillic Acid.
System: Ethylacetate M/10 Citrate buffer solution (pH 6.0)

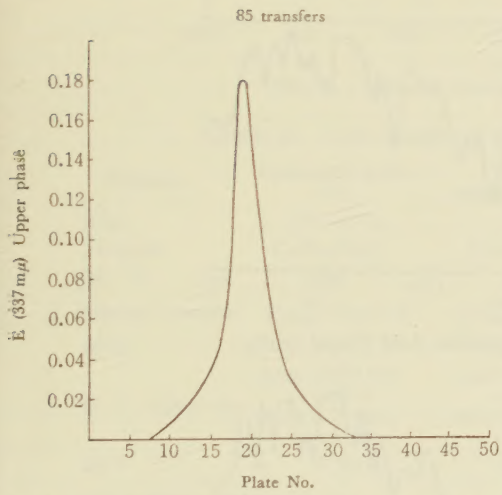

FIG. 3. Countercurrent Distribution Curve of Muta-aspergillic Acid (m.p. 174C°).
System: Ethylacetate, M/10 Citrate buffer solation (pH 6.2)

155°C was higher than those described in previous papers[1~3].

II) Physical and Chemical Properties of Muta-aspergillic Acid.

Muta-aspergillic acid is a pale yellow needle, m.p. 173~4°C (uncorrected), having a weak acidic character. It sublimes and is readily soluble in methyl alcohol, ethyl alcohol, *n*-butyl alcohol, ethyl acetate, butyl acetate, acetone, benzene and glacial acetic acid. Slightly soluble in ether, *n*-hexane, but practically insoluble in petroleum ether and ligroin. It is almost insoluble in cold water but soluble in alkaline solution and buffer solutions. $[\alpha]_D^{25}=0\pm0.3$ (C=0.35 in metha-

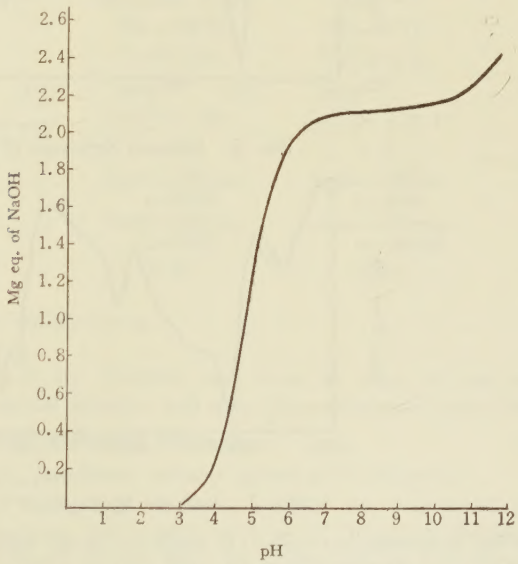

FIG. 4. Electronic Titration of Muta-aspergillic Acid with Glass Electrode.

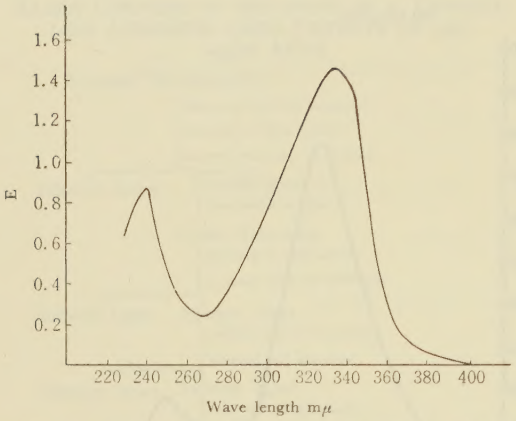

FIG. 5. Ultraviolet Absorption Spectrum of Muta-aspergillic Acid.

nol). Found: C 57.67; H 8.44; N 12.25% M.W. 246 (Rast method), 230 (titration); Calcd. for $C_{11}H_{18}H_2O_3$: C 58.41; H 7.96; N 12.39, M.W. 226. Kuhn-Roth C-methyl determination gave 1.9 moles of acetic acid per mole. pKa value for this antibiotic was determined by potentiometric titration as shown in Fig. 4, pKa=4.8. The ultra violet spectrum (Fig. 5) in methanol showed absorption peaks at λ_{max} 242 mμ (ε= 5100), λ_{max} 335 mμ (ϵ=8600). The infrared spectrum of this antibiotic is shown in Fig. 6. For comparison, the infrared spectra of hydroxyaspergillic acid, aspergillic acid* and neohydroxyaspergillic acid[4]** in nujol mull are shown in Fig. 7, 8 and 9, respectively.

The muta-aspergillic acid gives the positive ferric chloride test (red brown) and ninhydrin (red violet) reaction, but negative Fehling reaction. Primary amino group could not be observed by the microgasmetric method of Van Slyke. The R_F values on paper chromatography are as follows. By the ascending method for 18 hours at room temperature using Toyoroshi No. 50 and a solvent system of ligroin, pyridine, benzene, methanol and glacial acetic acid (70 : 30 : 6 : 3 : 1), muta-aspergillic acid, hydroxy-aspergillic acid neohydroxyaspergillic acid and aspergillic acid gave R_F values of 0.26, 0.45, 0.64 and 0.91 respectively as revealed by 2% aqueous ferric

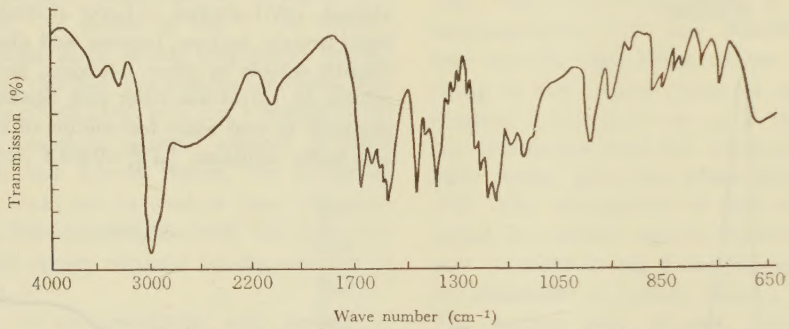

FIG. 6. Infrared Spectrum of Muta-aspergillic Acid (Nujol mull).

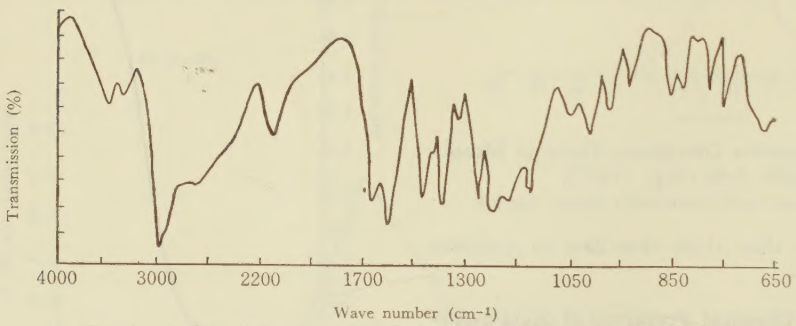

FIG. 7. Infrared Spectrum of Hydroxyaspergillic Acid (Nujol mull).

4) U. Weiss, F. Strelitz, H. Flow and I. N. Ascheshov, *Arch. Biochem. and Biophys.* **74**, 150 (1958).

* Kindly supplied by Dr. Dutcher.

** Kindly supplied by Dr. Weiss.

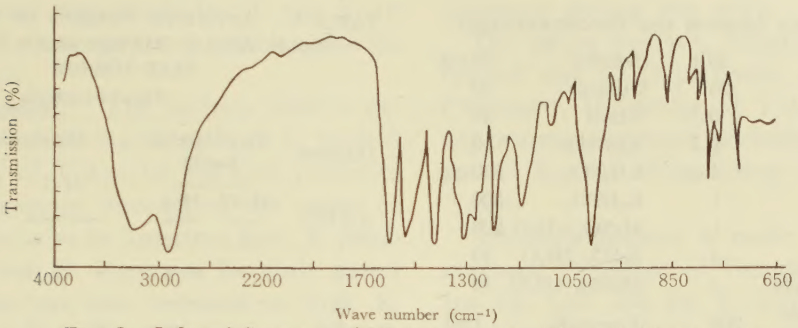

FIG. 8. Infrared Spectrum of Neohydroxyaspergillic Acid (Nujol mull).

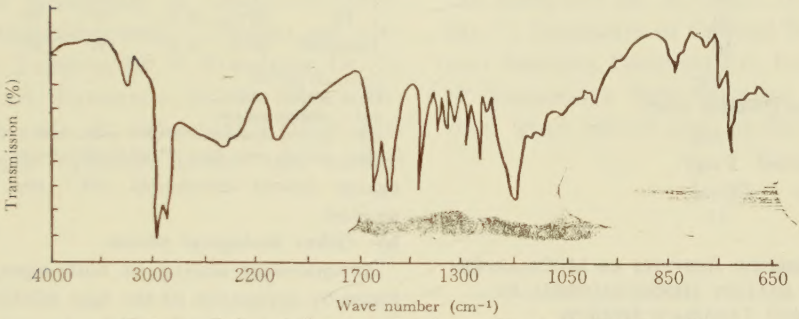

FIG. 9. Infrared Spectrum of Aspergillic Acid (Nujol mull).

TABLE II. COMPARISON OF PROPERTIES OF CYCLIC HYDROXAMIC ACIDS

Property	Muta-aspergillic Acid	Hydroxy-aspergillic Acid[1~3]	Neohydroxy-aspergillic Acid[4]	Aspergillic Acid[5~7]
M.p.	173~4°C	155°C	164~166°C	97~99°C
Formula	$C_{11}H_{18}N_2O_3$	$C_{12}H_{20}N_2O_3$	$C_{12}H_{20}N_2O_3$	$C_{12}H_{20}N_2O_2$
M. W.	226	240	240	224
Optical rotation	$[\alpha]_D^{25}=0\pm0.3°$	$[\alpha]_D^{28}=+36°$	$[\alpha]_D^{25}=-58°$	$[\alpha]_D^{24}=+13.4°$
pKa	4.8	4.9~5.1		5.5
U. V. Spectrum	$\lambda max=335\ m\mu$ $\varepsilon=8571$ $\lambda max=242\ m\mu$ $\varepsilon=5140$	$\lambda max=330\ m\mu$ $\varepsilon=7500$ $\lambda max=232\ m\mu$ $\varepsilon=7900$	$\lambda max=328\ m\mu$ $\varepsilon=8350$ $\lambda max=236\ m\mu$ $\varepsilon=6450$	$\lambda max=328\ m\mu$ $\varepsilon=8500$ $\lambda max=235\ m\mu$ $\varepsilon=10,500$
R_F*	0.26	0.45	0.64	0.91

* Filter paper: Toyôroshi No. 51.
Solvent: ligroin, pyridine, benzene, methanol, glatial acetic acid (70 : 30 : 6 : 3 : 1)

chloride solution. The properties of muta-aspergillic acid and related compounds are summarized in Table II for comparison.

As shown in Table II, the molecular formula of muta-aspergillic acid was found to be one methylene group less than the related compounds and moreover it is not identical with them in infra red spectra, optical rotation, and some physicochemical properties.

III) Biological Properties.

a) Antibiotic activity against hiochi-bacteria.

The growth of hiochi-bacteria was determined by titrating the acidity of 2 ml of Tamura's modified medium (Table III). Its activity against true hiochi-bacillus H-1 and hiochi-lactobacillis H-34 and H-7 was found to be bacteriostatic for true hiochi-bacillus H-1

5) E. C. White and J. H. Hill, *J. Bact.*, **45**, 433 (1943).
6) J. D. Dutcher, *J. Bioll. Chem.*, **171**, 321, 341 (1947).
7) G. Dunu, J. J. Gallagher, G. T. Newbold and F. S. Spring, *J. Chem., Soc.*, S 126, S 131 (1949); 2679 (1951).

TABLE III. ASSAY MEDIUM FOR HIOCHI-BACTERIA

B :	Glucose	20 g	Adenine	10 mg
	Na-acetate	20	Guanine	10
	Fumaric acid	0.5	Uracil	10
	NH_4Cl	0.5	Xanthine	10
	Vitamin-B_1	1 mg	KH_2PO_4	500 mg
	// B_2	1	K_2HPO_4	500
	// B_6	1	$MgSO_4 7 \cdot H_2O$	200
	Nicotic acid	1	$FeSO_4 \cdot 7H_2O$	10
	Pantothenic acid	1	$MnSO_4 \cdot 4H_2O$	10
	p-Aminobenzoic acid	200	Tween-80	1 ml
	Biotin	10		
	Folic acid	10		
	Vitamin-B_{12}	10		
P :	Polypeptone	5 g		
M :	Mevalonic acid (Hiochic acid)			
	natural	1 mg		
	(or synthesized	2 mg)		
S :	Japanese Sake	250 ml		
Total volume :		1 l		

TABLE IV. ANTIBIOTIC ACTIVITY OF MUTA-ASPERGILLIC ACID AGAINST HIOCHI-BACTERIA IN MODIFIED TAMURA'S MEDIUM

Medium dilution	B. P. M. (P : 0.5%)			B. S. (S , 0.5 ml/2 ml)		
($\times 10^4$)	H-1	H-7	H-34	H-1	H-7	H-34
1	0	0.54	0	0	0	0
2	0	0.70	0.16	0	0	0.12
4	0	2.04	3.39	0.02	2.06	1.37
8	0.06	2.08	3.70	0.30	2.27	2.45
10	0.42	2.15	3.69	0.49	2.09	2.43
16	0.77	2.23	3.64	0.81	1.93	2.73
20	0.80	2.32	3.40	0.83	1.89	3.07
∞	0.75	2.16	3.64	0.81	1.82	3.23

Note : In B. P. M., 5g of polypeptone (p) and 2 mg of mevalonic acid (M) were added to 1 l of basal medium (B).
In B. S., 250 ml of Japanese Sake (S) were contained in 1 l of basal medium (B).

at concentration of 1 : 40,000 and for hiochi-lactobacilli H-7 and H-34, at the concentration of 1 : 20,000 respectively (Table IV). In the case of Matsuyama's diluted Sake medium (composed of 70 parts Sake, 30 parts water, and 1 part polypeptone, containing alcohol 16~17%), the growth of hiochi-bacteria was determined by turbidity after 10 days incubation at 30°C. As shown in Table V, the minimum concentration for complete inhibition of growth against true hiochi-bacilli (*Lactobacillus homohiochii* H-42 and *Lactobacillus heterohiochii* H-1) was 5~10 mcg/ml, and

TABLE V. ANTIBIOTIC POTENCY OF MUTA-ASPERGILLIC ACID IN MATSUYAMA'S DILUTED SAKE MEDIUM

Dilution	Hiochi-bacteria: True hiochi-bacilli		Hiochi-lactobacilli			
			H-7		H-34	
($\times 10^4$)	H-42	H-1				
1	——	——	—	—	—	—
2	——	——	—	—	—	—
5	——	——	—	—	—	—
8	——	——	±	±	—	—
10	——	——	±	±	±	±
16	±±	±±	+	++	++	++
Controll	++	++	++	++	+++	+++

(—) : no growth.
(±) : slightly cloudy.
(+) : slightly growth.
(++) : growth more than 10^6/ml (viable count).
(+++) : growth more than 10^8/ml (viable count).

against hiochi lactobacilli (H-7 and H-34) 10~20 mcg/ml.

b) Other biological effects.

Antimicrobial activity of muta-aspergillic acid was tested by application of the agar dilution method, but the growth of *Bacillus subtilis, Staphylococcus aureus, Proteus vulgaris, Mycobacterium phlei, Mycobacterium* sp. 607, *Penicillium chrysogenum, Aspergillus niger, Candida albicans, Saccharomyces cereviciae, Torula utilis, Corynebacterium xerosis* was not inhibited even at a concentration of 1 : 1000.

c) Toxicity.

Toxicity of muta-aspergillic acid was tested with mice weighed 15~20 g. The LD_{50}, when administered intravenously was 100 mg/kg of body weight.

CONCLUSION

From the crude material of hydroxyaspergillic acid produced in the culture filtrate of *Aspergillus oryzae*, a new antibiotic named muta-aspergillic acid was isolated in pure state, which showed activity against true hiochi-bacilli and hiochi-lactobacilli.

Analysis and molecular weight determination agree with the formula $C_{11}H_{18}N_2O_3$. Although it is optically inactive, it has very similar chemical properties to those of hydroxyaspergillic acid.

The minimum concentration for complete inhibition of growth in a diluted Sake-peptone

medium against true hiochi-bacilli was 5~10 mcg/ml, and that against hiochi-lactobacilli 10~20 mcg/ml.

Acknowledgement. The authors wish to express their sincerest thanks to Prof. Y. Sumiki of University of Tokyo for his kind guidance and encouragement throughout the course of this work, and also to Emeritus Prof. K. Sakaguchi and Prof. H. Umezawa for their sound advice. They are also indebted to Prof. K. Kitahara of University of Tokyo, and Prof. S. Teramoto of University of Osaka for their cultures of hiochi-bacteria. Thanks are also due to Dr. S. Tamura, Dr. Y. Yonehara, Dr. G. Tamura, Dr. A. Kawarada, Dr. K. Miyao, Dr. N. Takahashi, Mr. K. Aizawa and Dr. S. Marumo for their valuable advice and cordial assistance during this work.

The micro analysis, infrared spectra were carried out at Department of Agricultural Chemistry, University of Tokyo and animal experiment tests were performed at the Institute of Applied Microbiology of University of Tokyo.

Acknowledgement is made to Mr. T. Yamamura, President of Yamamura Sake-Brewing Co. Ltd. and Dr. Y. Takeda of Sanraku Distillers Co. Ltd. for their encouragement in this work and Dr. A. Ozaki, Dr. N. Miyaji and Mr. Y. Kurimura of Central Research Laboratory Sanraku Distillers Co. Ltd. and members of Yamamura Sake-Brewing Laboratory for their kind advice and cordial advice.

Studies on Growth Inhibition of Hiochi-bacteria, Specific Saprophytes of Sake*

Part VII. Structure of Muta-aspergillic Acid (1)

By Seiji NAKAMURA

The outline of this report has already been published as a short communication[1] concerning muta-aspergillic acid, $C_{11}H_{18}N_2O_3$, a new growth inhibitant against hiochi-bacteria. Deoxymuta-aspergillic acid, $C_{11}H_{18}N_2O$, reduction product of muta-aspergillic acid, was converted to 2,5-diketopiperazine, $C_{11}H_{20}N_2O_2$, and this compound was shown to be leucyl-valine anhydride since on hydrolysis, it yielded leucine and valine. These results and physical and chemical data on muta-aspergillic acid led to draw the conclusion that formula (I) or (II) is the most possible structure for this antibiotic.

The author already reported the production of hydroxyaspergillic acid[2] as a growth inhibitant against hiochi-bacteria by *Aspergillus oryzae*.

In the previous paper[3] the author reported isolation of the other new growth inhibitant against hiochi-bacteria, named muta-aspergillic acid, from the pre-analysis material of hydroxyaspergillic acid.

The properties and infrared spectrum of muta-aspergillic acid suggested that this antibiotic belongs to the same group as aspergillic, hydroxyaspergillic and neohydroxyaspergillic acids having the same basic structure of cyclic hydroxamic acid. The molecular formula of muta-aspergillic acid, $C_{11}H_{18}N_2O_3$, was found to be less one methylene group than hydroxyaspergillic acid. Kuhn-Roth C-methyl determination gave 1.9 moles of acetic acid per mole. Hydroxyaspergillic acid contains an asymmetric carbon atom, but muta-aspergillic acid is optically inactive. Although the ultraviolet absorption spectrum of muta-aspergillic acid is very similar to that of hydroxyaspergillic acid, the peaks are shifted a little towards longer wave lengths.

Muta-aspergillic acid in methanol gave green crystalline copper salt, m.p. 231°C, with cupric acetate. The deep red coloration with ferric chloride and the formation of a green cupric salt suggest that this antibiotic might possess a hydroxamic acid group. The treatment of muta-aspergillic acid with acetic anhydride-pyridine gave crystalline mono-acetate, m.p. 110°C, $C_{11}H_{17}N_2O_3(CH_3CO)$ as readily as hydroxyaspergillic acid.

The infrared absorption bands at 3400 cm^{-1} (–OH) and 1120 cm^{-1} (–OH) in muta-aspergillic acid and hydroxyaspergillic acid disappeared in their mono-acetates (Fig. 1) but, the absorption band at 3200 cm^{-1} (–OH) of intermolecular hydrogen bonds did not vanish in both monoacetates. Moreover, these mono-acetates showed the positive ferric chloride

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1) S. Nakamura, Bull. Agr. Chem. Soc. of Japan, **24**, 629 (1960).
2) S. Nakamura and T. Shiro, *ibid.*, **23**, 65 (1959).
3) S. Nakamura and T. Shiro, Biol. Chem. **25**, 573 (1961).

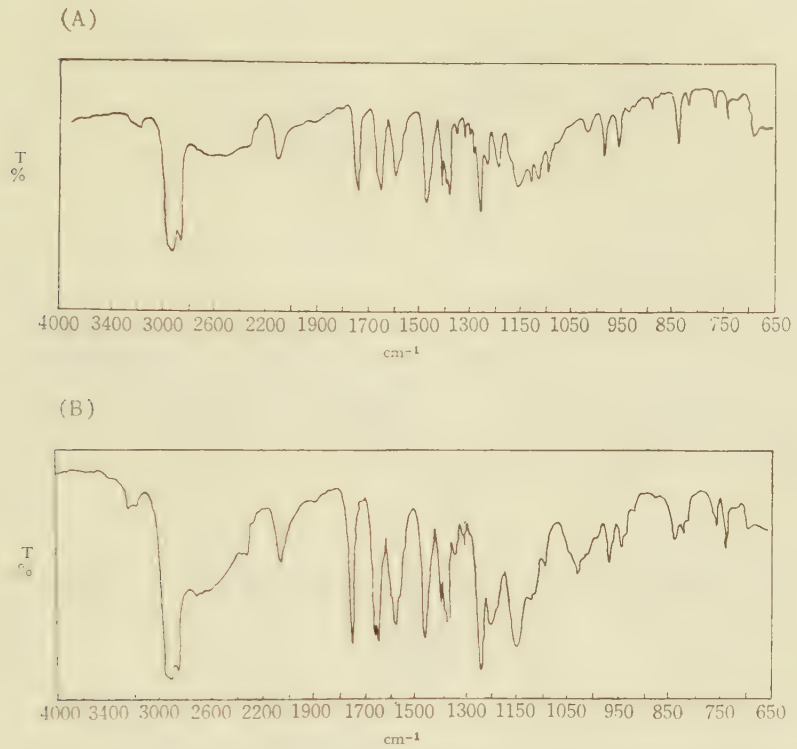
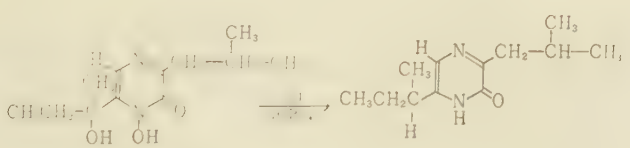


FIG. 1. Infrared Spectra of Monoacetate of Muta-aspergillic Acid (A) and Hydroxyaspergilic Acid (B) (nujol mull).

test, suggesting that the grouping –N(OH)–CO– of muta-aspergillic acid and hydroxyaspergillic acid could not be acylated under above mentioned condition. Thus the acetylable hydroxyl group of muta-aspergillic acid must be located on one of the side chains as in hydroxyaspergillic acid.

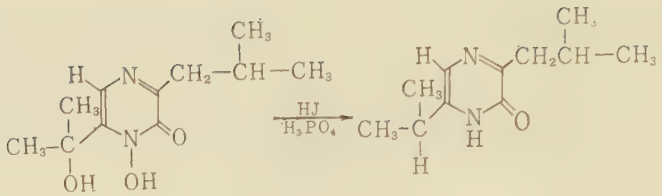
Dutcher[4] reported that the treatment of hydroxyaspergillic acid (III) with hydroiodic acid in phosphoric acid caused not only reduction of hydroxamic acid group to an amide but the reductive elimination of the hydroxyl-group in a side chain, yielding deoxyaspergillic acid (IV).



(III) (IV)

The similar reduction of muta-aspergillic acid gave a neutral compound, m.p. 106°C, $C_{11}H_{18}N_2O$ (V), named deoxymuta-aspergillic

4) J. D. Dutcher, *J. Biol. Chem.*, **232**, 785 (1958).



(I) (V)

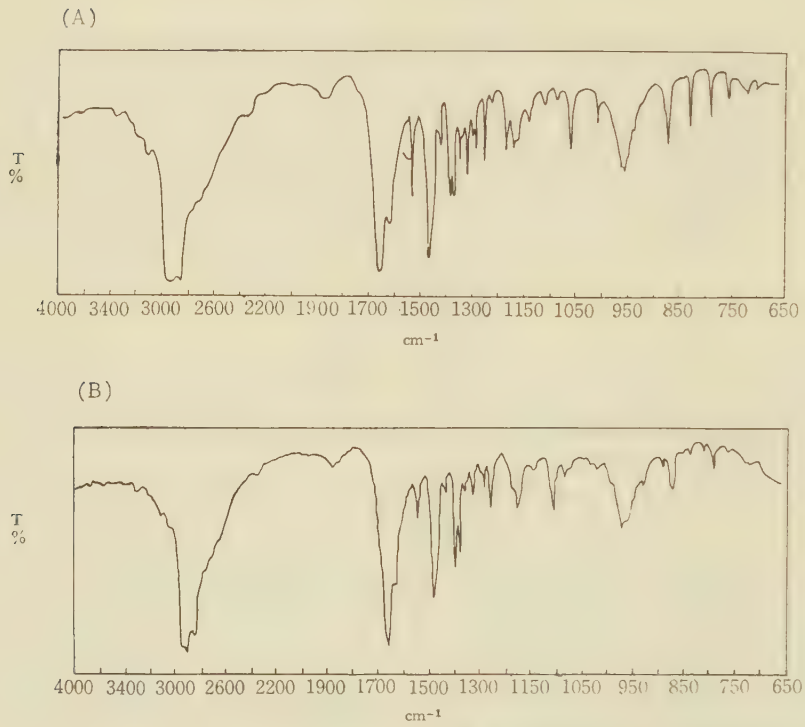


FIG. 2. Infrared Spectra of Deoxymuta-aspergillic Acid (A) and Deoxyaspergillic Acid (B). (Nujiol mull).

acid, which contained less one methylene group than deoxyaspergillic acid (IV), and showed negative ferric chloride reaction.

The infrared spectrum of this product shows complete disappearance of absorption bands of hydroxy groups as in the case of hydroxyaspergillic acid (Fig. 2). Its ultraviolet absorption spectrum was essentially identical with that of deoxyaspergillic acid (Fig. 3).

On the other hand, the reduction of hydroxyaspergillic acid with hydroiodic acid in acetic acid gave a neutral compound, m.p. 158°C, $C_{12}H_{18}N_2O$, called dehydrodeoxyaspergillic acid (VI), in which the hydroxamic group was reduced and one mole of water was eliminated from a hydroxyl group at a side chain.

However, in the case of muta-aspergillic acid, such an elimination of water did not

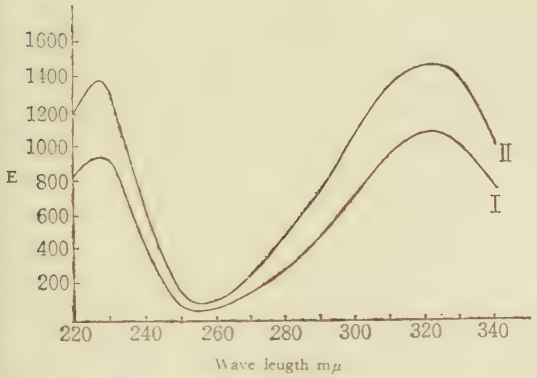


FIG. 3. Ultraviolet Spectra of Deoxymuta-aspergillic Acid and Deoxyaspergillic Acid.

I. Deoxyaspergillic acid. 167 γ/6 ml.
II. Deoxymuta-aspergillic acid. 256 γ/7.5 ml in methanol

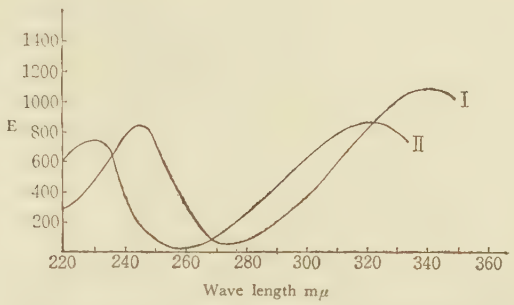
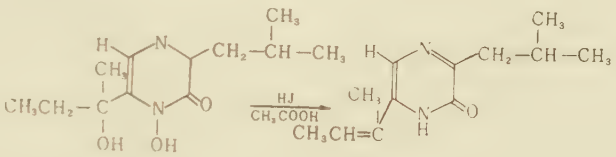


FIG. 4. Ultraviolet Spectra of Reduction Product of Hydroxyaspergillic Acid and Muta-aspergillic Acid by Hydroiodic Acid and Acetic Acid.

I. Dehydrodeoxyaspergillic acid. 183 γ/10 ml.
II. Deoxymuta-aspergillic acid. 205 γ/10 ml in methanol.



take place, and the resulting product was identified as deoxymuta-aspergillic acid, m.p. 108°C, $C_{11}H_{18}N_2O$ (V). Ultraviolet spectra of these both reduction products are shown in Fig. 4. By these comparisons between muta- and hydroxyaspergillic acid it can be concluded that muta-aspergillic acid have the structure closely related to hydroxyaspergillic acid.

In the same way as in the case of aspergillic acid[5,6], deoxymuta-aspergillic acid (V) was converted to 2,5-diketopiperazine compound, $C_{11}H_{20}O_2N_2$, m.p. 172~3°C (VIII) by the route shown in the following chart.

On hydrolysis with conc. HCl, this compound gave only two amino acids which were identified as leucine and valine by paper chromatography, proving that compound VIII should be 2,5-diketo-3-isobutyl-6-isopropyl-piperazine. Therefore deoxymuta-aspergillic acid must be either 3-hydroxyl-5-isobutyl-2-isopropyl pyrazine (X) or 3-hydroxyl-2-isobutyl-5-isopropyl pyrazine (IX).

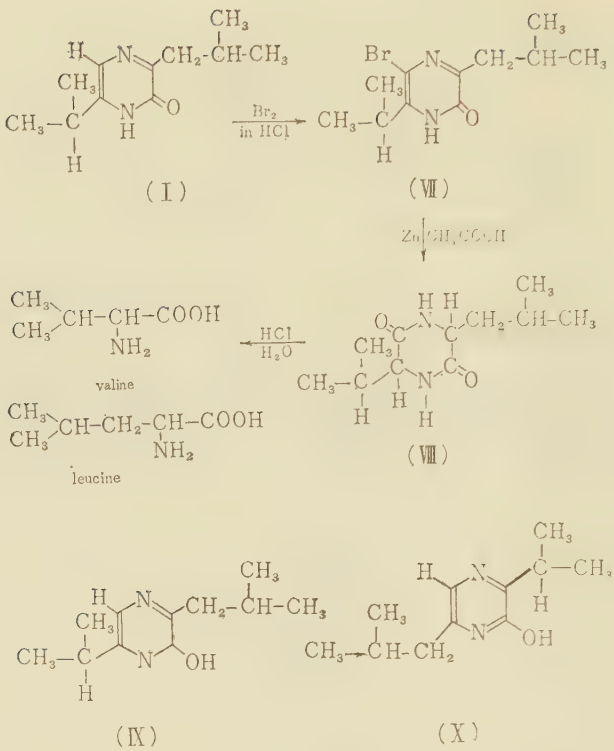
Moreover, it was concluded that the hydroxyl group is tertiary and is located at the isopropyl side chain from the following evidences.

(1) Since muta-aspergillic acid is optically inactive, asymmetric carbon atom is not contained in this compound.

(2) Muta-aspergillic acid does not form any iodoform on treatment with hypoiodite. This fact suggests that the group CH_3–CHOH– is not present in this compound.

(3) When hydroxyaspergillic acid was heated to 150°C in syruppy phosphoric acid, the acid was converted to dehydroaspergillic acid $C_{12}H_{18}N_2O_2$ as described by Dutcher,

5) G. Dunn, G. T. Newbold and F. S. Spring, *J. Biol. Chem. Soc.*, S. 131 (1949).
6) J. D. Dutcher, *J. Biol. Chem.*, 171, 321, 341 (1947).



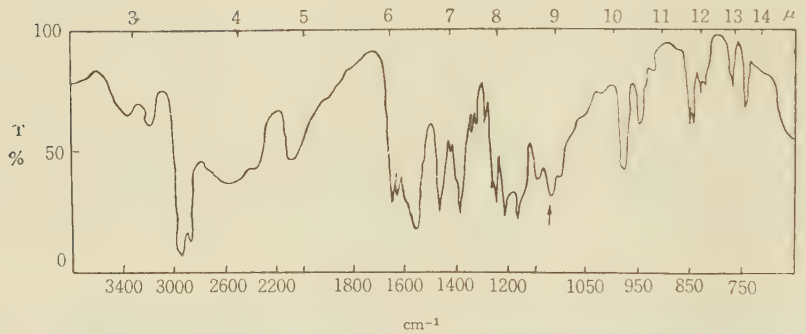
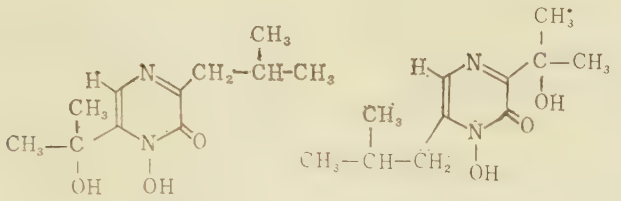


FIG. 5. Infrared Spectrum of Muta-aspergillic Acid (nujol mull).

but, in the case of muta-aspergillic acid, such a facile dehydration could not be observed.

(4) Infrared spectrum of muta-aspergillic acid (Fig. 5) shows the same absorption band at about 1120 cm^{-1}, as hydroxyaspergillic acid. It has been well established[7,8] in general, that tertiary alcohol has a C–O band at about 1150 cm^{-1} and this band is shifted about −30 cm^{-1} to longer wave length when α-carbon to tertiary alcohol has the branch. Therefore, this band at about 1120 cm^{-1} should be assigned to C–O stretching band of tertiary al-

7) K. Nakanishi, *Kagaku no Ryoiki*, **13**, 313 (1959).
8) H. Harold, H. Zeiss and M. Tsutsui *J. Am. Chem. Soc.*, **20**, 897 (1953).



(I) (II)

cohol group.

The above mentioned results lead to draw the conclusion that formula (I) or (II) is the most possible structure for muta-aspergillic acid.

EXPERIMENTAL

1) Muta-aspergillic Acid Monoacetate.

Thirty two mg of muta-aspergillic acid in pyridine and 0.5 ml of acetic anhydride were mixed under cooling. The mixture was kept for 24 hours at room temperature. And then, 1 ml of water was added. The pH was adjusted to 3.5 by adding HCl, and the mixture was extracted with chloroform. The chloroform layer was evaporated to dryness in vacuo, and crude crystals of monoacetate were obtained. On recrystallization from aqueous alcohol, about 15 mg of needles were obtained, m.p. 111°C.

Anal. Found: C, 58.01; H, 7.47; N, 10.42%. Calcd. for $C_{11}H_{17}N_2O_3 \cdot CH_3CO$: C, 58.21; H, 7.46; N, 10.45%.

2) Hydroxyasergillic Acid Monoacetate.

Fifty mg of hydroxyaspergillic acid were treated in the same way as in the previous case. About 35 mg of crude crystal of monoacetate were obtained. Recrystallization from aqueous alcohol yielded colorless needles, m.p. 106°C.

Anal. Found: C, 59.79; H, 7.74; N, 10.08%. Calcd. for $C_{12}H_{19}N_2O_3 \cdot CH_3CO$: C, 59.57; H, 7.80; N, 9.93%.

3) Reduction of Muta-aspergillic Acid with Hydroiodic Acid and Phosphoric Acid.

Fifty mg of red phosphorus and 0.05 ml of hydroiodic acid were added to a solution of 50 mg of muta-aspergillic acid in 1 ml of 85% phosphoric acid. The mixture was heated at 160°C for 3 hours. The hot solution was filtered through a sintered glass into 5 ml of 1 percent sodium bisulfite solution. The mixture was extracted with chloroform. The chloroform layer was evaporated in vacuo, and about 30 mg of crude crystal of deoxymuta-aspergillic acid were obtained. Recrystallization from acetone and *n*-hexane yielded colorless needles, m.p. 106°C.

Anal. Found: C, 67.80; H, 9.29; N, 14.26%. Calcd. for $C_{11}H_{18}N_2O$: C, 68.04; H, 9.28; N, 14.43%.

4) Reduction of Muta-aspergillic Acid with Hydroiodic Acid and Acetic Acid.

A solution of 50 mg of muta-aspergillic acid in 1 ml of glacial acetic acid was added to a solution containing 10 mg of iodine, 10 mg of red phosphorus and 1.5 ml of glacial acetic acid. The mixture was refluxed in water bath at 140°C for 2 hours and then filtered through a sintered glass into 15 ml of 1 percent sodium bisulfite solution. Precipitated needles were separated by filtration. Recrystallization from acetone yielded colorless needles, m.p. 108°C.

Anal. Found: C, 68.12; H, 9.40; N, 14.40%. Calcd. for $C_{11}H_{18}N_2O$: C, 68.04; H, 9.28; N, 14.43%.

Mixed melting point with authentic deoxymuta-aspergillic acid showed no depression, and infrared spectrum and ultraviolet spectrum (Fig. 4) were completely identical with those of deoxymuta-aspergillic acid.

5) Monobromodeoxymuta-aspergillic Acid.

A solution of 80 mg of deoxymuta-aspergillic acid in 3 ml of 5 N hydrochloric acid was treated with saturated bromine water until no further precipitate was formed. The colorless precipitate was separated by filtration. Recrystallization from aqueous alcohol yielded 30 mg of colorless needles, m.p. 113°C.

Anal. Found: C, 48.14; H, 6.43; N, 10.19%. Calcd. for $C_{11}H_{17}N_2OBr$: C, 48.35; H, 6.23; N, 10.26%.

6) 2, 5-Diketo-3-isobutyl-6-isopropylpiperazine.

Twenty-five mg of monobromodeoxymuta-aspergillic acid was dissolved in 0.5 ml of glacial acetic acid and 0.2 ml of water. To this solution 0.5 g of zinc dust was added and the mixture was heated in water bath for 3 hours. Four ml of water were added to this hot solution and the solution was kept in an ice box for

crystallization. Recrystallization from acetone yielded colorless needles, m.p. 173°C.

Anal. Found: C, 62.54; H, 9.45; N, 13.37%. Calcd. for $C_{11}H_{20}N_2O_2$: C, 62.26; H, 9.43; N, 13.20%.

7) Hydrolysis of 2, 5-Diketo-3-isobutyl-6-isopropylpiperazine.

Two mg of 2,5-diketo-3-isobutyl-6-isopropylpiperazine were hydrolysed with 1 ml of conc. HCl at 112°C for 40 hours in a small sealed tube. The reaction mixture was evaporated to dryness in vacuo and colorless needles of the mixture of hydrolysis product were obtained.

Hydrolysate was dissolved in about 0.2 ml of water and subjected to paper chromatography on Toyo filter paper No. 51, using a solvent system of tertiary amylalcohol saturated with water, and descending method for 20 hours at room temperature. For development of the spots, 0.2 per cent ninhydrin alcohol solution was used.

The amino acids in the hydrolysate were identified as leucine (R_F 0.32) and valine (R_F 0.15) by the comparison of R_F values of authentic amino acids.

Acknowledgement. The author wishes to express his sincere thanks to Prof. Y. Sumiki, University of Tokyo for his guidance and encouragement throughout the course of this work and to Emeritus Prof. K. Sakaguchi and Prof. H. Umezawa for their sound advices. Thanks are also due to Dr. S. Tamura, Dr. H. Yonehara, Dr. H. Kitamura, Dr. K. Miyao, Dr. Y. Hue and Mr. K. Aizawa of University of Tokyo, Dr. H. Shindo of Takamine Laboratory, Dr. A. Ozaki, Dr. N. Miyazi and Mr. Y. Kurimura of Central Research Laboratory, Sanraku Distillers Co. Ltd. for their valuable advices and cordial assistances in this work. The micro-analysis and infrared spectra were carried out at the Department of Agricultural Chemistry, University of Tokyo and the Institute of Applied Microbiology of University of Tokyo. The author also expresses his thanks to Sanraku Distillers Co. Ltd. for supplying the hydroxyaspergillic acid.

Acknowledgement is given to Mr. T. Yamamura, President of Yamamura Sake Brewing Co. Ltd. and Dr. Y. Takeda of Sanraku Distillers Co. Ltd. for their encouragement in this work.

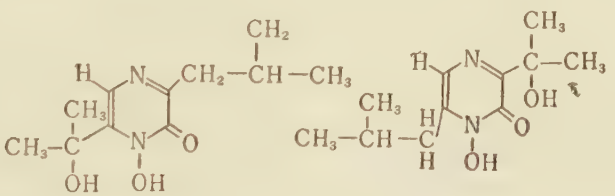
Studies on Growth Inhibition of Hiochi-bacteria, Specific Saprophytes of Sake*

Part VIII. Structure of Muta-aspergillic Acid (2)

By Seiji NAKAMURA

The outline of this report has already been published as a short communication[1] concerning the synthesis of deoxymuta-aspergillic acid and its identification with 2-isobutyl-5-isopropyl-3-hydroxypyrazine. This synthesis establishes the structure of muta-aspergillic acid as formula (I).

In the previous paper[2,3], the author reported the isolation of muta-aspergillic acid, $C_{11}H_{18}N_2O_3$, from the pre-analysis material of hydroxyaspergillic acid, and concluded that formula (I) or (II) is the most probable structure for this antibiotic.



(I)

(II)

This paper deals with the synthesis of 2-isobutyl-5-isopropyl-3-hydroxypyrazine, identity of which with deoxymuta-aspergillic acid was confirmed, establishing the final structure of muta-aspergillic acid as formula (I).

In the previous paper, it was pointed out that deoxymuta-aspergillic acid must be either 2-isobutyl-5-isopropyl-3-hydroxyprazine or 5-isobutyl-2-isopropyl-3-hydroxypyrazine. According to Newbold et al.[4] *rac*-deoxyaspergillic acid was identified as 2-isobutyl-5-sec-butyl-3-hydroxypyrazine by five steps synthesis starting from sec-butylglyoxal diethyl acetal.

Applying this synthetic method, 2-isobutyl-5-isopropyl-3-hydroxypyrazine was synthesized via the following procedure from isopropylglyoxal diethyl acetal (VII). The starting material, isopropylglyoxal diethyl acetal was prepared by the same route as reported by H. D. Dakin et al.[5]. Isopropylglyoxal diethyl acetal (VII) was treated with hydroxylamine

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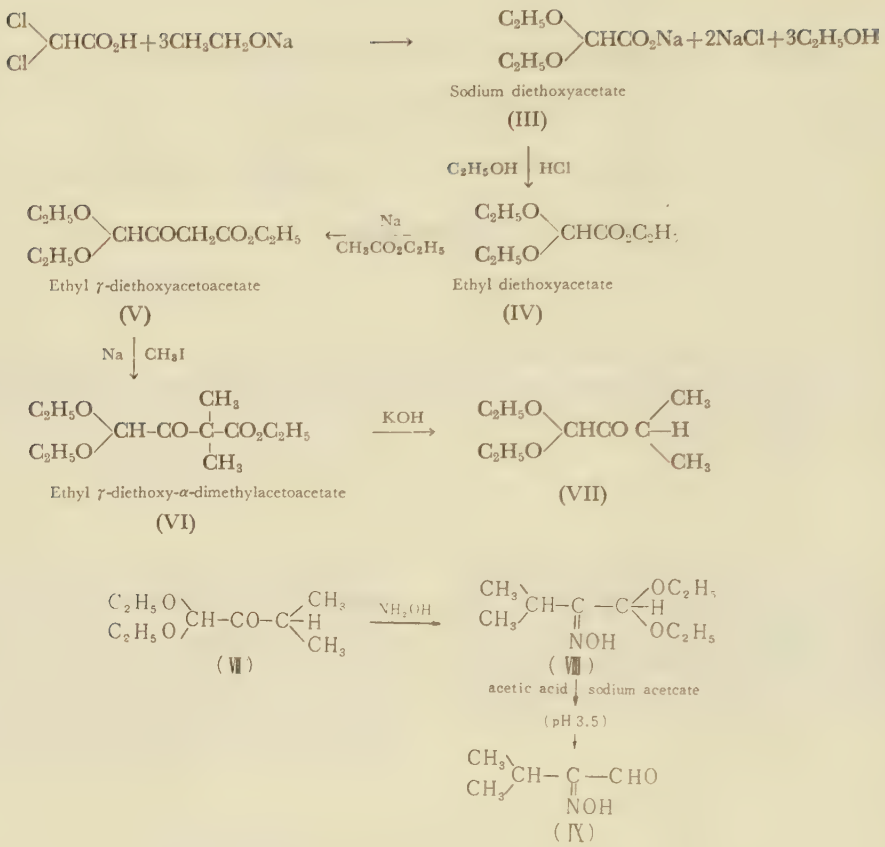
1) S. Nakamura, Agr. Biol. Chem. **25**, 74 (1961).
2) S. Nakamura and T. Shiro, *ibid.*, **25**, 573 (1961).
3) S. Nakamura, *ibid.*, **25**, 658 (1961).
4) G. T. Newbold, W. Sharp and F. Springs, *J. Chem. Soc.* 2679 (1951).
5) H. D. Dakin, and H. W. Duddley, *ibid.*, 2453 (1914).



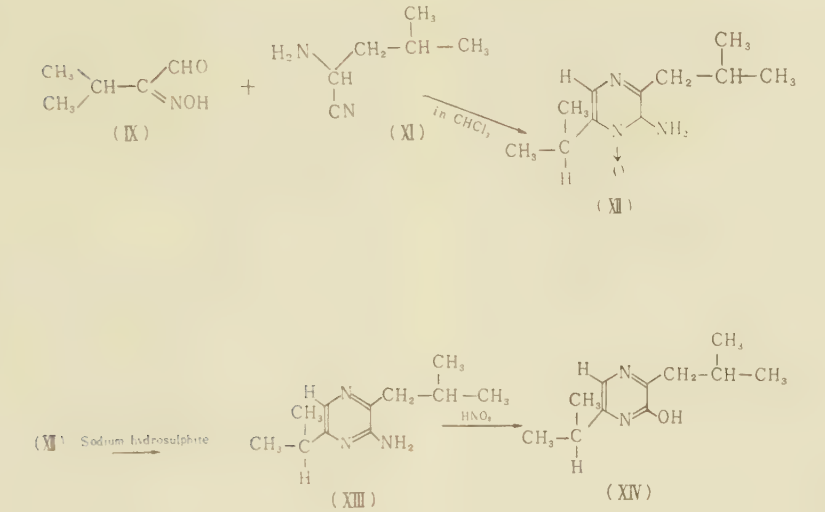
to yield α-oximino-isovaleraldehyde diethyl acetal (VIII), from which α-oximino-isovaleraldehyde (IX) was prepared by hydrolysis with acetic acid-sodium acetate solution (pH 3.5).

Condensation of α-amino-γ-methylvaleronitrile (XI) with α-oximino-isovaleraldehyde (IX) yielded 2-amino-3-isobutyl-6-isopropylpyrazine-1-oxide (XII).

Reduction of (XXII) with sodium hydro-

On the other hand, α-amino-γ-methylvaleronitrile (DL-leucine nitrile) (XI) was obtained by the treatment of isovaleraldehyde (X) with ammonium chloride and potassium cyanide by the method of G. T. Newbold et al.[4]

Reduction of (XII) with sodium hydropyrazine (XIII). This amino-pyrazine was converted to 2-isobutyl-5-isopropyl-3-hydroxypyrazine (XIV) by treatment with nitrous acid.



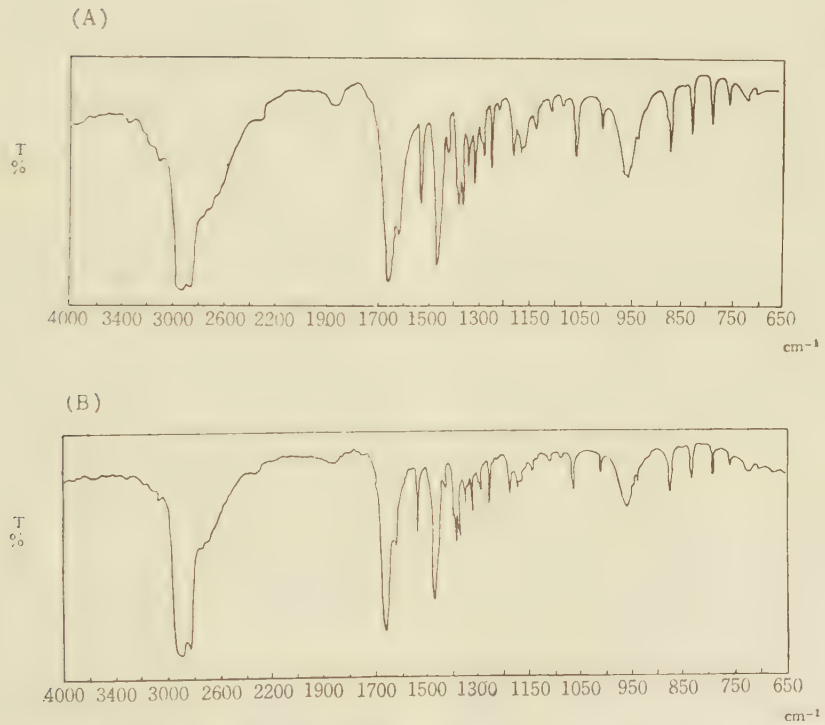


FIG. 1. Infrared Spectra of Deoxymuta-aspergillic Acid (A) and Synthesized 2-isobutyl-5-isopropyl-3-hydroxypyrazine (B).

The identity of synthesized 2-isobutyl-5-isopropyl-3-hydroxypyrazine (XIV) with deoxymuta-aspergillic acid was confirmed by mixed melting point and comparison of infrared spectra (Fig. 1).

Evidence that the hydroxyl group of muta-aspergillic acid is tertiary and locates at the isopropyl side chain was described in a previous paper[3], and then the structure of muta-aspergillic acid was clearly established as shown in (I).

EXPERIMENTAL

1) Synthesis of Isopropylglyoxal Acetal.

i) Ethyl diethoxyacetate.

Sodium ethoxide was prepared by adding 62 g of sodium in portions to 900 ml of absolute alcohol. When practically all the sodium was dissolved, 100 g of dichloroacetic acid was added with stirring. After the initial reaction subsided, the reaction mixture was heated under reflux for 3.5 hours. Then the solution was cooled below 0°C. After cooling with stirring, a solution of about 55 g hydrogen chloride in 400 ml of absolute alcohol was slowly added. The mixture was allowed to come to room temperature, and stirring was continued for 3 hours. It was then allowed to stand overnight. The reaction mixture was again cooled to 0°C, and the excess acid was neutralized to pH 7 by slowly adding sodium ethoxide solution. The mixture was then filtered, and the precipitate was extracted throughly with ether which was added to the alcoholic filtrate. The solid was discarded. The solvent was removed by distillation under reduced pressure. Ethyl diethoxyacetate was obtained by distillation under diminished pressure as colorless liquid, b.p. 67~69°C/10~8 mm. The yield was 61 g.

Anal. Found: C, 54.58; H, 9.15. Calcd. for $C_8H_{16}O_4$: C, 54.54; H, 9.09%.

ii) Ethyl γ-diethoxyacetoacetate.

Sixty g of ethyl diethoxyacetate were heated to 85~90°C in an oil bath, and 24 g of metallic sodium and 91 g of ethyl acetate were added slowly in seventeen portions. After the reaction subsided, 40 ml of alcohol was added to the mixture, and the residual metallic sodium was dissolved in it. Then, the reaction mixture was poured into the mixture of 100 g of conc. hydrochloric acid and 100 g of ice. Oily layer was separated and collected. Aqueous layer was extracted with 50 ml of ether. Ether layer and oily layer were combined and washed with aqueous sodium carbonate. Ether and alcohol were distilled off in vacuo, and 57 g of ethyl γ-diethoxyacetoacetate were obtained by distillation under diminished pressure, b.p. 109~112°C/6 mm.

Anal. Found: C, 55.23; H, 8.36. Calcd. for $C_{10}H_{18}O_5$: C, 55.04; H, 8.25%.

iii) Ethyl γ-diethoxy-α-dimethylacetoacetate.

Sodium ethoxide from 6 g of sodium in 92 ml of alcohol, 57 g of ethyl γ-diethoxyacetoacetate and 42.5 g of methyl iodide were mixed, and gently boild for 30 minutes in a water bath. Then, to this mixture, sodium ethoxide solution prepared from 6 g of sodium and 92 ml of alcohol, and 48 g of methyl iodide were added, and the whole mixture was boiled for 30 minutes. Most of the solvent was removed by distillation. Adding 20 ml of water, the residue was extracted with ether repeatedly. The solvent layer was combined and dried on anhydrous sodium sulphate. After removal of the solvent, the residue was distilled under reduced pressure to yield 51.5 g of ethyl γ-diethoxy-α-dimethylacetoacetate, b.p. 118°C/10 mm.

Anal. Found: C, 58.55; H, 8.56. Calcd. for $C_{12}H_{22}O_5$: C, 58.54; H, 8.94%.

iv) Hydrolysis of ethyl γ-diethoxy-α-dimethylacetoacetate.

Eighteen g of ethyl γ-diethoxy-α-dimethylacetoacetate were dissolved in 115 ml of methanol and were hydrolysed with same volume of 2 N potassium hydroxide solution under reflux for 30 minutes. After the addition of 100 ml of water, the reaction mixture was extracted with five 50 ml portions of ether. Ether extracts were combined and dried on anhydrous sodium sulphate. After removal of the solvent, the residue was distilled under reduced pressure to yield 11 g isopropylglyoxal acetal, b.p. 60°C/6 mm.

Anal. Found: C, 62.10; H, 10.42. Calcd. for $C_9H_{18}O_3$: C, 62.06; H, 10.34%.

2) Synthesis of α-Oximino-isovaleraldehyde.

i) α-Oximino-isovaleraldehyde diethyl acetal.

The mixture of 7.2 g of isopropylglyoxal acetal, 60 ml of methanol, 29 ml of water and 3.1 g of anhydrous sodium carbonate was stirred well at room temperature. A solution of 2.96 g of hydroxylamine hydrochloride in 14.4 ml of water was added to this mixture. The reaction mixture was stirred throughly

for 26 hours at room temperature and gave the pH below 8. After the removal of methanol in diminished pressure, the residue was extracted with four 50 ml portions of ether. The ether layer was combined and dried over anhydrous sodium sulphate. Then, ether was removed in vacuo, and 6.2 g of α-oximino-isovaleraldehyde diethyl acetal were obtained by distillation under diminished pressure as colorless oil, b.p. 106～7°C/5 mm.

Anal. Found: C, 56.98; H, 10.29; N, 7.45. Calcd. for $C_9O_{19}O_2N$: C, 57.14; H, 10.05; N, 7.41%.

ii) Hydrolysis of α-oximino isovaleraldehyde diethyl acetal.

Six g of α-oximino-isovaleraldehyde diethyl acetal were dissolved in 45 ml of methanol. Forty five ml of acetate buffer solution (1 M acetic acid and 0.1 M sodium acetate; pH 3.5) were added to the solution, and the mixture was refluxed at 100°C for 4 hours in an oil bath. Then, the mixture was cooled to room temperature and methanol was removed in vacuo. After adding 45 ml of water, this reaction mixture was extracted with four 25 ml portions of ether. The ether extracts were combined, and dried on anhydrous sodium sulphate. Then the solvent was removed in vacuo, and 2.2 g of α-oximinoisovaleraldehyde were obtained by distillation under diminished pressure as an oily product, b.p. 50～52°C/0.3 mm.

Anal. Found: C, 52.95; H, 8.42; N, 12.42. Calcd. for $C_5H_9O_2N$: C, 52.17; H, 7.82; N, 12.17%.

3) Synthesis of Dexymuta-aspergillic Acid.

i) Condensation of α-oximino-isovaleraldehyde and DL-leucine nitrile hydrochloride.

Twenty five ml of chloroform, 6.65 g of DL-leucine nitrile hydrochloride and 4.95 g of 4-methyl morpholine were poured into a separatory funnel with vigorous agitation. Then a solution of 5.2 g of α-oximino-isovaleraldehyde in 7.5 ml of chloroform was added and the mixture refluxed for 4 hours in an oil bath. The reaction mixture was allowed to come to room temperature and filtered. The filtrate was evaporated in vacuo. The residue was dissolved in 30 ml of ether and extracted with four 15 ml portions of 3 N hydrochloric acid. Aqueous layers were combined and extracted with two 10 ml portions of ether. The ether extracts were neutralized to pH 8 with sodium hydroxide, and extracted with four portions of 50 ml ether. Solvent layers were combined and dried over anhydrous sodium sulphate. After removal of ether, the residue was distilled under reduced pressure to yield 590 mg of 2-amino-3-isobutyl-6-isopropylpyrazine-1-oxide as an orange color oil, b.p. 130°C/0.25 mm.

Anal. Found: C, 62.92; H, 9.29; N, 19.33. Calcd. for $C_{11}H_{19}N_3O$: C, 63.15; H, 9.09; N, 20.09%.

ii) Reduction of 2-amino-3-isobutyl-6-isopropyl-pyrazine-1-oxide

Five hundred and seventy mg of 2-amino-3-isobutyl-6-isopropylpyrazine-1-oxide were dissolved in 14 ml of 50% aqueous ethyl alcohol. Adding 0.9 g of sodium hydrosulphite to this solution, the mixture was boiled for 30 minutes in an oil bath under reflux. Then, twenty 2.5 ml portions of alcohol, 0.25 ml of water and 0.4 g of sodium hydrosulphite were added in a period of 20 hours. After the reaction subsided, alcohol was removed by distillation in vacuo, and the residue was made alkaline by the addition of 3 N sodium hydroxide solution and extracted with four 30 ml portions of ether. The combined extract was washed with 15 ml of distilled water and dried over anhydrous magnesium sulphate. After filtration and removal of the ether by distillation, amber colored oily residue was dissolved in 25 ml of alcohol. By the addition of saturated alcohol solution of picric acid yellow felted needles of 3-amino-2-isobutyl-5-isopropylpyrazine picrate were crystallized out. Recrystallization from alcohol 41 mg of crystals, m.p. 121～2°C, were obtained.

Anal. Found: C, 47.63; H, 5.19; N, 19.17. Calcd. for $C_{11}H_{19}N_3$ ($C_6H_3O_7N_3$) : C, 48.34; H, 5.21; N, 19.9%.

iii) 2-Isobutyl-5-isopropyl-3-hydroxy-pyrazine.

3-Amino-2-isobutyl-5-isopropylpyrazine picrate (390 mg) was dissolved in 5 ml of hot ethanol. After the addition of 10 ml of 3 N hydrochloric acid to this solution, the alcohol was distilled off in vacuo. The residue was dissolved in 10 ml of water and extracted with two 10 ml portions of ethyl acetate to remove picric acid. The aqueous layer was evaporated in vacuo to dryness. The solid residue was dissolved in 5 ml of 3 N hydrochloric acid and cooled to 0°C. Then 0.25 g of sodium nitrite was slowly added in portions over 5 minutes with stirring. After the reaction mixture was kept at 0°C for 30 minutes and heated to 50～60°C for five minutes, it was neutralized to approximately pH 7 by adding sodium carbonate, and extracted with two 5 ml portions of 2 N potassium hydroxide solution. When the aqueous layer was neutralized by adding 3 N hydrochloric acid, colorless precipitate crystallized out.

After recrystallization from aqueous alcohol 30 mg of 2-isobutyl-5-isopropyl-3-hydroxypyrazine (m.p.

109°C) were obtained.

Anal. Found: C, 67.90; H, 9.29; N, 14.34. Calcd. for $C_{11}H_{18}N_2O$: C, 68.04; H, 9.28; N, 14.43%.

Acknowledgement. Author wishes to express his thanks to Prof. Y. Sumiki of University of Tokyo for his guidance and encouragement throughout the course of this work and to Emeritus Prof. K. Sakaguchi and Prof. H. Umezawa for their sound advices.

Author is also indebted to Dr. S. Tamura, Dr. H. Yonehara, Dr. K. Miyao, Dr. Y. Hsu, Mr. M. Takai, Mr. K. Aizawa of Department of Agricultural Chemistry, University of Tokyo for their valuable advices and kind help in this work. The microanalysis, infrared spectrum were carried out at Department of Agricultural Chemistry of University of Tokyo.

Last acknowledgement is expressed to Mr. Y. Yamamura, President of Yamamura Sake Brewing Co. Ltd. and Dr. Y. Takeda of Sanraku Sake Brewing Co. Ltd.

Studies on the Proteolytic Enzymes of Black *Aspergilli**

Part VI. Further Studies on the Specificity of Crystalline *Aspergillus Saitoi* Protease

By Fumihiko Yoshida and Eiji Ichishima

In order to determine the specificity of *Aspergillus Saitoi* protease, the hydrolyzate of B-chain of insulin oxidized by this enzyme was investigated on paperchromatography according to the 2,4-dinitrofluorobenzene technique. Specificity was compared with pepsin and other proteolytic enzymes.

Several groups of workers have reported on the specificity of the proteolytic enzymes towards the synthetic peptides and the modified protein. In a previous paper of this series[1], a study has been made to determine the specificity of the action of this enzyme toward the oxidized lysozyme through analysis of N-terminal amino acids residues by application of Sanger's DNP-technique.

In this paper, the B-chain of the oxidized insulin[2~4] was used for study in detail of specificity of this enzyme. Since the B-chain of insulin is a single peptide having an known-structure and does not contain any amount of tryptophan, this was considered very useful to serve for the study of specificity of the proteolytic enzyme. The DNP-peptides from enzymatic hydrolyzate were separated and the amino acid composition was identified paperchromatographically.

EXPERIMENTAL

Substrate: Beef insulin (Simizu Seiyaku Co. Ltd.) was oxidized by performic acid[5] and the oxidized products were separated by Sanger's precipitation method[6]. The yield of B-chain of oxidized-insulin was 13.2%. The N-terminal amino acid residue of the separated B-chain was identified paperchromatographycally in the same way as that of phenylalanine.

Enyzme: Crystalline *Asp. Saitoi* protease was prepared according to the method of Yoshida et al.[7]

Preliminary estimation of enzymrtic degradation by *Asp. Saitoi* protease and pepsin: Two mg of the substrate dissolved in 10 ml of acidic water was digested by *Asp. Saitoi* protease (pH 2.8) and pepsin (pH 2.0). After 24 hr, the enzymatic reaction was stopped by heat-treatment and the degree of degradation was estimated by the colorimetric ninhydrin method of Cocking and Yemm[8].

Condition of enzymatic reaction: Fifty mg of the substrate was dissolved in 20 ml of acidic solution (pH 2.8), mixed with 0.5 ml of *Asp. Saitoi* protease solution, and allowed to react at 37°C for 24 hours. After boiling for 3 min so as to inactivate the enzyme, the reaction mixture was lyophilized.

Indentificat of peptides from *Asp. Saitoi* protease hydrolyzate: The 2,4-dinitrophenylation of the enzymatic hydrolyzate was carried out by the same method as described in the previous paper[3]. The

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1) F. Yoshida, M. Nagasawa and E. Ichishima, Bull. Agr. Chem. Soc. of Japan, **23**, 363 (1959).
2) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 463, 481 (1951).
3) F. Sanger, E.O.P. Thompson and R. Kitai, *ibid.*, **59**, 509 (1955).
4) A.P. Ryle, F. Sanger, F. Smith and R. Kitai, *ibid.*, **60**, 541 (1955).
5) F. Sanger and E.O.P. Thompson, *Biochem. J.*, **53**, 353 (1953).
6) F. Sanger, *ibid.*, **44**, 126 (1949).
7) F. Yoshida and M. Nagasawa, Bull. Agr. Chem. Soc. of Japan, **22**, 32 (1958).
8) L.C. Cocking adn E.W. Yemm, *Biochem. J.*, **53**, xii (1954).

FIG. 1-a. Paperchromatogram of DNP-Peptides B-Chain Hydrolyzate

DNP-peptides were extracted with ether and ethylacetate, and each of the extracted fractions and acid soluble residues was developed on two dimensional paperchromatography. The solvents used in the paperchromatography procedure were as follows:

for ether or ethylacetate soluble fractions;

tert-Amylalcohol—N/10 KH-phthalate buffer (pH 6.9) saturated.

1.5 M Phosphate buffer (pH 6.0).

for the water soluble fraction;

n-Butanol-1% ammonia (1 : 1, v/v).

1.5 M Phosphate buffer (pH 6.0).

The number of spots of DNP-peptides obtained on each chromatogram of the three fractions was as follows: 13 from the ether fraction, 5 from the ethylacetate fraction, and 6 from the water fraction. The spots were cut off and extracted at 40~50°C with acetic acid, N-hydrochloric acid or 1% sodium bicarbonate. The extracted solution of DNP-peptides was evaporated in vacuo subsequently hydrolyzed with 6 N-hydrochloric acid in a sealed tube at 110~115°C for 12 hours. The amino acid composition of the hydrolyzed solution was determined by paperchromatography.

The following chromatographic solvents were used:

for ether soluble DNP-amino acids;

tert-Amylalcohol system and 1.5 M phosphate buffer (two dimensional).

n-Butanol-*n*-buthylacetate—1% ammonium hydroxide (1 : 2 : 3 v/v)[9]. (for separation of DNP-Leu and DNP-Val)

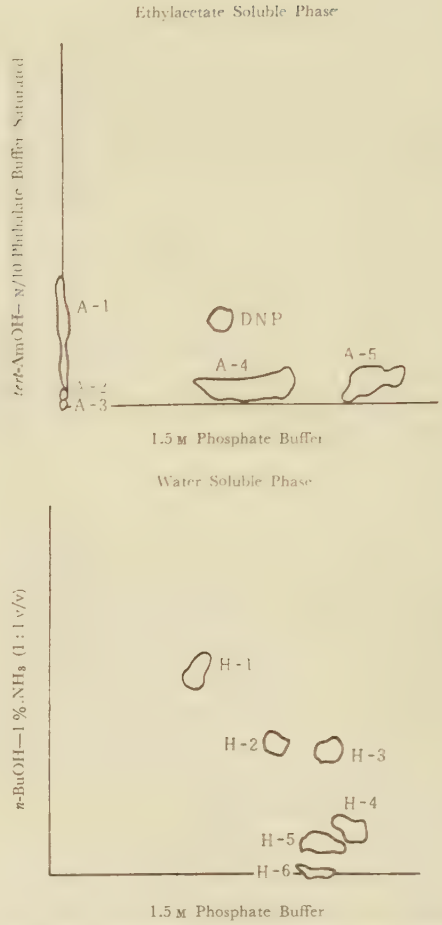


FIG. 1-b. Paperchromatogram of DNH-Peptides from B-Chain Hydrolyzate

for water soluble DNP-amino acids:

n-Butanol system and 1.5 M phosphate buffer (two dimensional).

tert-Butanol—formic acid—water (70 : 1 : 30, v/v).

and, for identification of amino acids:

tert-Butanol—formic acid system.

Phenol—N/10 KH-phthalate buffer (pH 6.0, 3 : 1, v/v) 0.1% α-benzoinoxime.

RESULT AND DISCUSSION

Typical data obtained from preliminary ex-

9) E.F. Mellon, A.H. Korn and S.R. Hoover, *J. Am. Chem. Soc.*, **75**, 1675 (1953).

periments in which the degrees of hydrolysis of B-chain of insulin oxidized by *Asp. Saitoi* protease and by pepsin determined by the colorimetric ninhydrin method are shown in Table I. From the table it is apparent that the degree of enzymatic digestion by this enzyme is higher than pepsin, and that decomposition of the substrate by heat-treatment is negligible in each case.

In recent years, protein chemistry has born out an expection that the ion exchange chromatography procedure can possess the highest general resolving power surpassing any method so far developed for the separation of peptides and amino acids from protein hydrolyzate. However, Davies et al.[10] have demonstrated that the cleavage of insulin both by the cation exchange resin Amberlite IR-120 (acid form) and by the anion exchange resin Amberlite IR-4B (hydroxyl form) mainly occurs at temperature in the portions of the B-chain near to the carboxyl end and outside the ring bounded by the disulfide bridge. For this reason, the paperchromatographic method was applied to separate the enzymatic hydrolyzate under a most mild condition as possible.

The kind of peptides thus separated are listed in Table II. The separation of some peptides was incomplete and some spots were

TABLE I. ESTIMATION OF ENZYMATIC DEGRADATION BY *Asp. Saitoi* PROTEASE

Pepsion Hydrolyzate	O.D.	Ratio of Degradation
0 hr.	0.269	0 %
24	0.357	16.2
24, and Boil	0.360	16.8
NaOH-Hydrolsis	0.812	100
Asp. Saitoi Protease Hydrolyzate		
0	0.336	0
24	0.512	25.1
24, and Boil	0.521	26.7
NaOH-Hydrolysis	1.037	100

10) J.W. Davies and G. Harris, *Arch, Biochem. Biophys.*, **74**, 229 (1958).

a mixture composed of peptides. However since the substrate used here was a single polypeptide having a known structure, the amino acid sequence of these separated peptides could be easily supposed. In this experiment, lysin was recognized as a free amino acid in the hydrolyzate.

After comparing 30-minutes' and 24-hours' digestion it was proved that Ala, Leu, Phe, Tyr and Val appear as N-terminal amino acid residues at 30-minutes' digestion, whereas besides the above amino acids, Glu, Glu-NH_2, Lys, Arg, $CySO_3H$. His are observed at 24-hours' digestion considered to be caused by the action of *Asp. Saitoi* protease.

Fig. 2 summarizes the specificity of *Asp. Saitoi* protease and those of other proteolytic enzymes on B-chain of oxidized insulin. It thus appears that our enzyme has a considerable wider range of specificity than trypsin[2], chymotrypsin[2], parapepsin-I and -II[11] and cathepsin-D[12]. On comparison of the specificity of pepsin[2] with that of our enzyme on the modified protein, it seemed that most of the bonds in the following sequences Glu NH_2·His(4∼5), Leu·Val(11∼12), Gly·Ala·Leu·Tyr·Leu(13∼17) and Phe·Tyr(25∼26) are susceptible to hydrolysis. The bonds Phe·Val(1∼2) and Gly·Phe·Phe·Tyr(23∼26) are split by Sepsin exclusivly, while our enzyme specifically cleaved the bond in the following manner. AspNH_2·GluNH_2(3∼4), Leu·$CySO_3H$·(6∼7), His·Leu (10∼11), $CySO_3H$·Gly·Glu·Arg (19∼22) and Pro·Lys·Ala (28∼30). Apart from this, it can be said that both pepsin and our enzyme split those bonds adjacent to Ala, Leu, Phe, Tyr residues, and the cleavage by both enzymes mainly occurs in the interior of the ring portion which is bound by the two cysteic acids or $CySO_3H$ (7) and $CySO_3H$ (19). Besides such action, it was demonstrated that

11) A.D. Ryle and R.R. Porter, *Biochem. J.*, **73**, 75 (1959).
12) E.M. Press, R.R. Porter and J. Cebra, *ibid.*, **74**, 501 (1960).

Table II. Peptides form *Asp*.

Spot No.	Phe. 1	Val. 2	$AspNH_2$. 3	$GluNH_2$. 4	His. 5	Leu. 6	$CySO_3H$. 7	Gly. 8	Ser. 9	His. 10	Leu. 11	Val. 12	Glu. 13
E–8, E–10	Phe.	Val.	$AspNH_2$.	$GluNH_2$.									
E–4, E–5, E–7	Phe.	Val.	$AspNH_2$.	$GluNH_2$.	His.	Leu.							
E–1, A–1, A–2, A–3	Phe.	Val.	$AspNH_2$.	$GluNH_2$.	His.	Leu.	$CySO_3H$.						
E–3	Phe.	Val.	$AspNH_2$.	$GluNH_2$.	His.	Leu.	$CySO_3H$.	Gly.	Ser.	His.			
A–4				$GluNH_2$.	His.	Leu.	$CySO_3H$.	Gly.	Ser.	His.	Leu.	Val.	
E–6					His.	Leu.							
H–4							$CySO_2H$.	Gly.	Ser.	His.			
H–5, H–6							$CySO_2H$.	Gly.	Ser.	His.	Leu.		
A–2							$CySO_2H$.	Gly.	Ser.	His.	Leu.	Val.	
H–1							$CySO_2H$.	Gly.	Ser.	His.	Leu.	Val.	Glu.
E–9											Leu.	Val.	Glu.
E–6, H–3												Val.	Glu.
E–10, E–11, E–12, E–13, A–5												Val.	Glu.
E–13													Glu.
E–6													
E–8													
E–5													
H–5, H–6													
H–2													
H–4													
E–2													
A–3													
E–1, A–1													
A–1, A–2, A–3													
E–3													

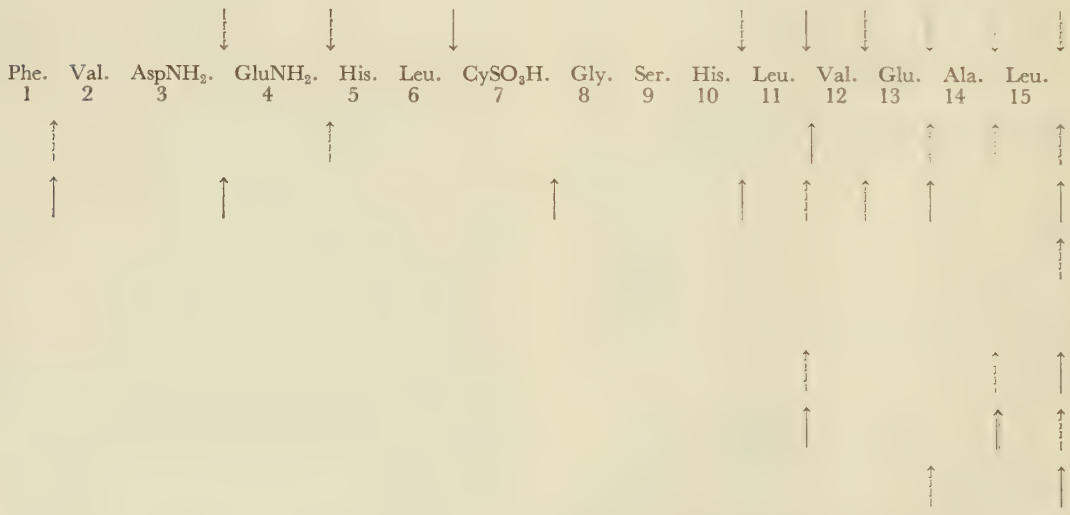
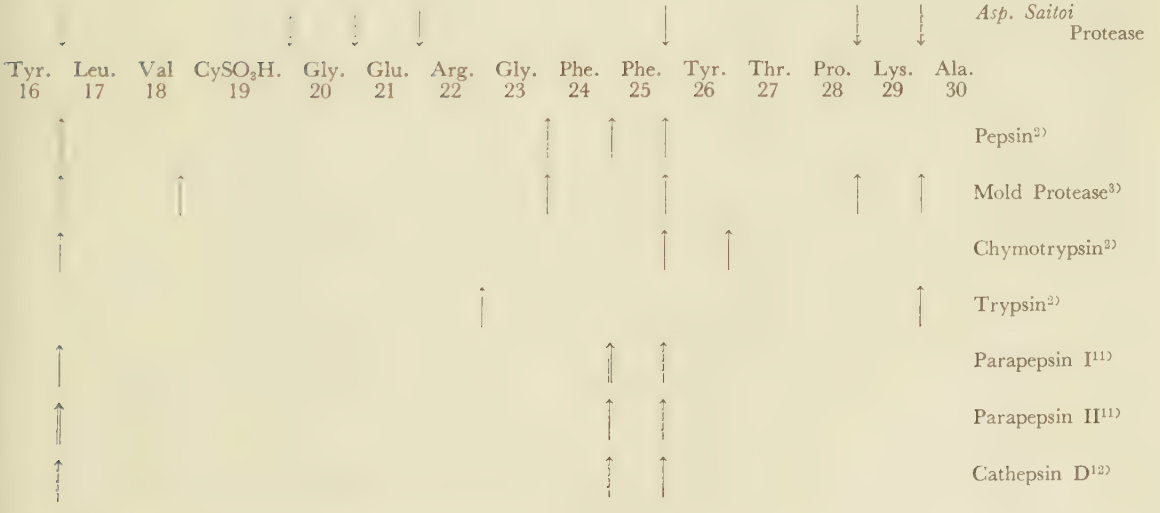


Fig 2. Site of Cleavage of B—Chain of Oxidized

Saitoi PROTEASE HYDROLYZATE

Ala.	Leu.	Tyr.	Leu.	Val.	$CySO_3H$.	Gly.	Glu.	Arg.	Gly.	Phe.	Phe.	Tyr.	Thr.	Pro.	Lys.	Ala.
14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30

Ala.	Leu.	Tyr.	Leu.	Val.												
Ala.	Leu.	Tyr.	Leu.													
Ala.	Leu.	Tyr.	Leu.	Val.	$CySO_3H$											
Ala.																
Ala.	Leu.	Tyr.	Leu.													
Ala.	Leu.	Tyr.	Leu.	Val.	$CySO_3H$.											
	Leu.	Tyr.	Leu.	Val.	$CySO_3H$.											
	Leu.	Tyr.	Leu.	Val.	$CySO_3H$.	Gly.	Glu.									
		Tyr.	Leu.	Val.	$CySO_3H$.	Gly.	Glu.	Arg.	Gly.	Phe.	Phe.	Tyr.	Thr.	Pro.	Lys.	Ala
			Leu.	Val.	$CySO_3H$.	Gly.	Glu.	Arg.	Gly.	Phe.	Phe.					
						Gly.	Glu.									
							Glu.	Arg.	Gly.	Phe.	Phe.					
								Arg.	Gly.	Phe.	Phe.	Tyr.	Thr.	Pro.	Lys.	Ala.
												Tyr.	Thr.	Pro.	Lys.	Ala.
															Lys.	



Insulin by *Asp Saitoi* Protease and Other Enzyme.

Asp. Saitoi protease splits most of the portion outside of the ring which is formed by the above two cysteic acids, and that further splitting by our enzyme take place at site of N-terminal residues of the basic amino acid, such as arginine, histidine and lysin. These facts exhibit distinct differences of proteolytic action between *Asp. Saitoi* protease and pepsin.

The mould protease (pH 8.0) reported by Crewther et al.[13], which Sanger et al. consider to be extremely active though rather unspecific than pepsin, parapepsin-I, -II, trypsin and chymotrypsin. It is very interesting to find that microbial protease generally have a considerably wider range of specificity than the animal protease.

SUMMARY

1. The B-chain of oxidized insulin was hydrolyzed with crystalline *Asp. Saitoi* protease and the enzymatic hydrolyzates were separated on paperchromatography according to the 2,4-dinitrofluorobenzene technique.

2. It was revealed that *Asp. Saitoi* protease has a considerably wider range of specificity than pepsin, parapepsin-I, -II, trypsin and cathepsin-D. By comparing pepsin with *Asp. Saitoi* protease, it can be said that both enzymes split the bond adjacent to alanine, leucine, phenylalanine and tyrosine residues. Besides such action it was found that splitting of our enzyme occurs at N-terminal residues of the basic amino acids.

Acknowledgement We wish to express our thanks to Dr. K. Sakaguchi, Institute of Physical and Chemical Research, Prof. B. Maruo, University of Tokyo, for their constant guidance. Our thanks are also due to Dr. M. Mogi and members of this laboratory for their encouragement in this work.

13) W.G. Crewther and F.G. Lennox, *Nature*, **165**, 680 (1950).

Studies on Osmophilic Yeasts*

Part XI. Various Factors Affecting on Polyalcohol Production by *Pichia miso*

By Hiroshi Ōnishi, Narimasa Saito and Ikunori Koshiyama

Various conditions were studied which gave influences on polyalcohol production by *Pichia miso*. *Pichia miso* gave an excellent yield of polyalcohol showing good growth in the vitamin-free medium. Unlike *Zygosaccharomyces* high concentrations of phosphate such as 2% as KH_2PO_4 in the medium showed no detrimental effect on polyalcohol production. Remarkable reduction in polyalcohol yield was observed in the medium of high concentration of nitrogen sources, among which yeast extract showed the most striking effect. Polyalcohol fermentation was provoked in the medium of 0.1% yeast extract while the metabolic activity distinctly converted to ethanol fermentation when the organisms were incubated in the medium of 4.0% yeast extract. The fact that a large amount of ethanol more than 6% in the medium was produced aerobically by genus *Pichia* of oxidative dissimilation type seemed to be very interesting and noticeable. For the purpose of industrial production, it was shown that polyalcohol production in jar-fermenter scale was achieved with as good yield as that in shaking flask culture.

INTRODUCTION

In the previous paper[1], one of the authors reported that a highly osmophilic yeast, *Pichia miso* Mogi[2], which was isolated from miso paste could produce glycerol, ertyhritol and D-arabitol at a very high yield, 50~60% of sugar consumed as total polyalcohols. It should be noted that this yeast was able to dissimilate completely highly concentrated glucose such as 30%, producing much polyalcohols as those stated above and that high concentration of glucose in the medium such as 30~40% was most preferable for the production of these polyalcohols.

Spencer et al.[3] has reported that phosphate content of the medium gives a significant influence on polyalcohol production by *Zygosaccharomyces* sp. i.e. in the medium containing more than 0.03% Na_2HPO_4, yields of polyalcohols are remarkably reduced. Peterson et al.[4] has stated that low levels of both inorganic phosphate and yeast extract favor the polyalcohol production by *Zygosaccharomyces*. More recently, Hajny et al.[5] observed similar phenomena in glycerol production by *Torulopsis magnoliae*.

In the present paper, influences of various cultural conditions on the production of polyalcohols by *Pichia miso* will be described. It was also shown that polyalcohol production in jar-fermenter scale was achieved with a good

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1) H. Ōnishi, Bull. Agr. Chem. Soc. of Japan, **24**, 131 (1960).

2) M. Mogi, *J. Agr. Chem. Soc. Japan*, **15**, 921, 1023, 1221 (1939), **16**, 7, (1940).

3) J.N.T. Spencer and P. Shu, *Can. J. Microbiol.*, **3**, 559, (1957).

4) W.H. Peterson, W.F. Hendershot and G.J. Hajny, *Appl. Microbiol.*, **6**, 349 (1958).

5) G.J. Hajny, W.F. Hendershot and W.H. Peterson, *Appl. Microbiol.*, **8**, 5 (1960).

yield as well as in the shaking flask culture.

In our experiment employing *Pichia miso*, phosphate concentrations of the medium had no effect on the polyalcohol yields at all i.e. in the medium containing as much as 2% KH_2PO_4, reduction in the polyalcohol yield could not be observed. C:N ratio of the medium had a great influence on polyalcohol yield, and especially in the medium of high concentration of yeast extract such as 2~4%, ethanol fermentation occurred instead of polyalcohol fermentation.

EXPERIMENTAL

Experimental methods employed in this study were the same as those described in the previous report[1], modifications being made with respect to the kinds and concentrations of nitrogen sources, salts and vitamins in the medium.

RESULTS

(1) Influences of omission of inorganic salts and vitamines from the medium.

The simple media consisting only of glucose and various nitrogen sources were employed for the incubation of *Pichia miso*. In these media, according to the deficiency in nutrients sugar consumptions were very poor compared with the ordinary medium hitherto employed. (Table I)

Table I. Polyalcohol Production by *Pichia miso* in the Simple Media Without Salts and Vitamins

Components of media	% of glucose consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on glucose consumed (as glycerol)
glucose 30% ordinary medium (control)*	29.60	12.35	41.7%
glucose 30% and yeast ext. 0.2%	9.42	4.65	49.3%
glucose 30% and polypeptone 0.2%	2.62	0.85	32.8%
glucose 30% and ammonium lactate 0.2%	4.27	1.61	37.7%
glucose 30% and casamino acid 0.4%	4.58	0.52	11.3%

* glucose 30%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCl_2 \cdot 2H_2O$ 0.01%, Vitamin-free Casamino acid (Difco) 0.4%, yeast ext. 0.1%

(2) Influence of omission of vitamins from the medium.

As stated in the previous paper[6], *Pichia miso* showed good growth even in the vitamin-free medium. Influence of vitamins on its polyalcohol production was examined. No significant difference was observed between the ordinary and the vitamin-free media. (Table II)

(3) Influence of phosphate

It was demonstrated by the previous result[1] that the addition of salts to the medium was essential for the polyalcohol production by *Pichia miso*. The effect of individual salt was examined in the following experiments.

Spencer et al.[3] and Peterson et al.[4] reported that polyalcohol production by *Zygosaccharomyces* favored a very low concentration of phosphate in the medium. A quite different result was obtained in the case of *Pichia miso* as shown in Table III. In the media containing less than 0.01% KH_2PO_4, the sugar consumption was extremely poor and subsequently the amount of polyalcohol produced was small. Good yield was obtained in the media containing more than 0.1% KH_2PO_4. Unlike *Zygosaccharomyces* even as much as 2%

Table II. Effect of Omission of Vitamins on Polyalcohol Production by *Pichia miso*

Media	% of glucose consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on glucose consumed (as glycerol)
ordinary medium	28.36	12.47	43.9%
vitamin-free medium*	28.17	12.34	43.8%

* as a nitrogen source, vitamin-free casamino acid (Difco) was employed

6) H. Ōnishi, Bull. Agr. Chem. Soc. of Japan, **21**, 143 (1957).

TABLE III. EFFECT OF PHOSPHATE CONCENTRATION OF THE MEDIUM ON POLYALCOHOL PRODUCTION BY *Pichia miso*

KH_2PO_4 concentration in the basal medium*	% of glucose consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on glucose consumed (as glycerol)
0	0.45	0.20	44.4%
0.01%	11.84	4.32	36.4%
0.1 %	23.16	11.10	47.9%
1.0 %	29.09	13.42	46.1%
2.0 %	29.47	14.04	47.6%

* Basal medium : glucose 30%, Ammonium lactate 0.4%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCl_2 \cdot 2H_2O$ 0.01%, NaCl 0.01%

KH_2PO_4 did not show any detrimental effect on the polyalcohol production.

(4) **Influences of magnesium and calcium salts.**

In the ordinary medium[1] concentrations of $MgSO_4 \cdot 7H_2O$ and $CaCl_2 \cdot 2H_2O$ were 0.05% and 0.01% respectively. From 10 to 50 times high concentrations of these salts did not reduce the polyalcohol yield. (Table IV)

(5) **Influences of nitrogen concentrations of the medium.**

As shown in the previous paper[1], the yields of polyalcohol were similarly good in every

TABLE IV. EFFECT OF Mg^{++} AND Ca^{++} CONCENTRATION OF THE MEDIUM ON POLYALCOHOL PRODUCTION BY *Pichia miso*

Media	% of glucose consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on glucose consumed (as glycerol)
Ordinary medium*	28.17	12.34	43.8%
$MgSO_4 \cdot 7H_2O$ 0.5%	29.79	12.90	43.3
$MgSO_4 \cdot 7H_2O$ 2.0%	29.02	12.38	42.6
$CaCl_2 \cdot 2H_2O$ 0.05%	29.59	12.41	41.9
$CaCl_2 \cdot 2HO_2$ 0.5%	30.10	12.63	41.9

* The same as that in Table I.

TABLE V. EFFECT OF NITROGEN CONCENTRATION OF THE MEDIUM ON POLYALCOHOL PRODUCTION BY *Pichia miso*

nitrogen sources and its concentration in the basal media*		% of glucose consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on glucose consumed (as glycerol)
Casamino acid	0.1 %	9.52	4.58	48.1 %
	0.4 %	31.28	13.64	43.6
	1.0 %	29.73	9.09	30.5
	2.0 %	31.28	7.50	24.0
	4.0 %	19.73	6.90	34.9
yeast extract	0.5 %	30.31	12.97	42.7 %
	1.0 %	29.70	8.81	29.6
	2.0 %	27.72	3.39	12.2
	4.0 %	22.99	1.85	8.0
L-glutamic acid	0.1 %	11.46	6.05	52.7 %
	0.4 %	26.61	13.47	50.6
	1.0 %	25.01	11.33	45.3
	2.0 %	26.61	10.29	38.6
	4.0 %	25.99	5.00	19.2
ammonium lactate	0.1 %	10.26	4.99	48.6 %
	0.4 %	30.10	13.95	46.3
	1.0 %	23.65	7.72	32.6
	2.0 %	24.11	8.12	33.6
	4.0 %	11.23	1.55	13.8

* The same as that in Table III except ammonium lactate ; 0.1% KH_2PO_4 was supplemented.

Table VI. Conversion Phenomenon Between Polyalcohol Fermentation and Ethanol Fermentation in *Pichia miso*

concentration of yeast extracts in the basal medium**	% of glucose consumed	% of ethanol produced	yield of ethanol on glucose consumed*	% of polyacohol produced (as glycerol)	yield of polyalcohol on glucose consumed (as glycerol)
yeast ext 0.1 %	27.18	0.09	0.6 %	13.32	49.0 %
yeast ext 4.0 %	18.60	6.10	64.1 %	1.14	6.1 %

* This values was conventionally calculated from Gay Lussac formula of ethanol fermentation.
** The same as that in Table V.

case of NH_3-N and NH_2-N as sole nitrogen of the medium, provided the pH value of the medium was suitable. In this paper, effect of nitrogen concentration in the medium has been examined.

As the sole source of nitrogen, casamino acid, L-glutamic acid, ammonium lactate or yeast extract was employed. The range of its concentration was between 0.1 and 4.0%. From the results shown in Table V, remarkable fall in polyalcohol yield in spite of much sugar consumption was observed in the medium of high nitrogen concentration. Although this tendency was seen in all cases of four nitrogen sources, yeast extract showed the most striking eeffct. It became clear that C : N ratio of the medium affects significantly on the polyalcohol production by *Pichia miso*.

In order to extend the study on this phenomenon, polyalcohol and ethanol determination in the two kinds of fermented broth, of 0.1% yeast extract and 4.0% yeast extract was conducted. As shown in Table VI, typical polyalcohol fermentation was provoked in the ordinary medium containing 0.1% yeast extract whereas the microbial activity converted to the ethanol fermentation when 4.0% yeast extract was supplemented. The fact that a large amount of ethanol more than 6% in the medium was produced aerobically by genus *Pichia* of film-forming, oxidatively dissimilating type, seemed to by very interesting and should be noticed. The mutual conversion between polyalcohol fermentation and ethanol fermentation may be a very important finding in view of the switch-over of the fermentation pathways and the close relation of the both fermentation mechanisms.

(6) Influence of addition of a large amount of vitamins.

The effective components in yeast extract were unknown which provoked the remarkable conversion of fermentation pathways as shown in the above experiments. Yeast extract

Table VII. Effect of High Concentrations of Vitamins on Polyalcohol Production by *Pichia miso*

Media		% of glucose consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on glucose consumed (as glycerol)
Basal medium*		28.17	12.32	43.8 %
//	+vitamins (×10**)	29.50	12.89	43.6 %
//	+vitamins (×50**)	30.07	13.42	44.6 %
//	+vitamins (×100**)	29.11	12.77	43.8 %
//	+thiamine 400γ/100cc	21.72	7.96	36.6 %
//	+thiamine 4 mg/100 cc	32.05	12.95	40.4 %
//	+thiamine 40 mg/100 cc	26.91	9.82	36.4 %
//	+thiamine 400 mg/100 cc	26.91	9.92	37.6 %

* glucose 30%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCl_2 \cdot 2H_2O$ 0.01%, vitamin-free casamino acid (Difco) 0.4%.
** added eight vitamins as 10~100 times as amount of Lodder and Kreger-van Rij's medium[6].

TABLE VIII. INFLUENCE OF TRACE ELEMENT

Media	% of glucose consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on glucose consumed (as glycerol)
Basal medium*	28.26	14.26	50.4 %
〃 +mixture of trace element**(1)	30.47	14.67	48.1
〃 + 〃 (2)	28.99	15.35	52.9
〃 + 〃 (3)	19.31	10.05	52.0

* glucose 30%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCl_2 \cdot 2H_2O$ 0.01%, vitamin-free casamino acid (Difco) 0.4%
** The amounts of added trace elements per 100 ml of the medium were as follows:

	mixture (1)	mixture (2)	mixture (3)
H_3BO_3	500γ	5 mg	50 mg
$CuSO_4 \cdot 5H_2O$	40	0.4	4
KI	100	1	10
$FeCl_3 \cdot 6H_2O$	200	2	20
$MnSO_4 \cdot 4H_2O$	400	4	40
$Na_2MoO_4 \cdot 2H_2O$	200	2	20
$ZnSO_4 \cdot 7H_2O$	400	4	40

TABLE IX. SELECTION OF PRACTICAL MEDIA FOR INDUSTRIAL PRODUCTION OF POLYALCHOL

Media	% of glucose consumed	% of polyalcohol produced (as glycerol)	yield of polyalcohol on glucose consumed (as glycerol)
glucose 30%, raw soy-sauce 0.5%	6.33	1.98	31.2 %
〃 〃 1.0	11.40	4.92	43.1
〃 〃 2.0	23.68	10.14	42.8
〃 〃 5.0	29.30	8.40	28.6
〃 〃 10.0	29.30	6.18	21.1
〃 〃 20.0	24.50	6.25	25.5
〃 soy bean cooked liquor 5%	17.01	9.00	52.9
〃 〃 10	25.10	13.13	52.3
〃 〃 20	29.53	11.97	40.5
〃 〃 30	29.53	11.97	40.5
〃 〃 40	12.74	1.65	12.9
〃 〃 50	14.44	1.65	11.4

(Difco Laboratory) used in these experiments contained no enzymatic activity[7]. An influence of addition of large amounts of vitamins on the above conversion was studied. Amounts of added vitamins were 10~100 times as those employed in Lodder and Kreger-van Rij's medium[8]. Meanwhile thiamine, which seemed important for the metabolism as a component of the coenzyme of carboxylase, was also examined at high concentrations. As shown in Table VII, no remarkable reduction in polyalcohol yield was observed in the medium of high concentrations of vitamins.

7) C.W. Christensen, Personal communication.
8) J. Lodder and N.J.W. Kreger-van Rij, The Yeasts a taxonomic study (1952).

(7) Influence of trace elements.

Effect of relatively high concentrations of trace elements such as B, Cu, I, Fe, Mn, Mo and Zn on polyalcohol production was examined. As shown in Table VIII, no appreciable change in polyalcohol production was observed.

(8) Selection of practical media for industrial production of polyalcohol.

Selection of economical media for industrial production of polyalcohol was carried out. The simple media consisting of 30% glucose and 2% raw soy-sauce, or of 30% glucose and 10~20% soy bean cooked liquor were found satisfactory giving excellent polyalcohol yield

as the complete medium hitherto employed. (Table IX)

(9) Polyalcohol production in jar-fermenter scale.

6 l of the medium consisting of 30% glucose, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2 \cdot 2H_2O$, and 0.4% casamino acid, were sterilized in a 15 l standard type jar-fermenter and 160 ml of 24 hour-shaking culture of *Pichia miso* were inoculated in the medium. The culture was aerated with 10~15 l per minute of sterile air and agitated of 500 r.p.m. 2~4 ml of defoaming agents (soy bean oil containing 2% silicon or soy-sauce oil) were added every 3 hours as soon as the fermentation started. Unless this defoaming process was carried out, the sugar consumption became fairly poor owing to vigorous growth of yeast cells on the surface of the foam. After 48 hour's fermentation, the forming settled and favorable submerged growth proceeded. It was also necessary for getting good results to inoculate fresh shaken culture of *Pichia miso* every 2~3 days.

After 10 days' incubation, the fermentation products were subjected to analysis[9,10]. As shown in Table X, satisfactory results were obtained in two separate experiments and the formation of glycerol, D-arabitol and ertyhritol was ascertained according to paper-chromatography[11,12].

Table X. Polyalcohol Production in Jar-fermenter Scale

	Experiment I	Experiment II
% of glucose consumed	26.90	25.44
% of polyalcohol produced (as glycerol)	11.10	12.13
yield of polyalcohol on glucose consumed (as glycerol)	41.2%	47.6%

(10) True ethanol determination of the fermentation broth by partition chromatography.

In the experiment described previously (part V), it was revealed that a large amount of ethanol more than 6% was produced in the medium of high concentration of yeast extract aerobically by *Pichia miso*. In that case, value determined as ethanol might contain other neutral voltatile products since all ethanol, acetone, diacetyl, isopropanol and *n*-butanol were recovered in the distillate in the determination procedure. True ethanol was determined after separating from other neutral volatitle compounds by chromatography on celite water column using CCl_4 and $CHCl_3$ as developing solvent according to the method of Neish[9,10].

94.2% of the value previously determined as ethanol was recovered in ethanol fraction of partition chromatography. Consequently, it was sure that *Pichia miso* produced aerobically much ethanol in the medium of high concentration of yeast extract.

(11) Identification of ethanol as ethyl-3-5-dinitro benzoate.

The ethanol in the fermented broth was identified which was obtained by shaking culture of *Pichia miso* in the medium containing 4% yeast extract. 3-5-dinitrobenzoate derivative as prepared with the fractional distillate of the fermented broth at 78°C according to the method of N. D. Cheronis and J. B. Entrikin[13].

The melting point of ethyl-3-5-dinitrobenzoate obtained was 93.5°C. This crystal did not depress the melting point of admixture with an authentic specimen which was prepared from special grade ethanol.

Anal. Found: C: 45.61%, H: 3.40%, N: 11.90%, Calcd. for $C_9H_8O_3N_2$: C: 45.01%, H: 3.36%, N: 11.67%.

Therefore, it was confirmed that ethanol was aerobically produced in the medium of

9) A.C. Neish, Analytical methods for bacterial fermentation 2nd rev. Natl. Research Council. Can., Praire Regional Lab., Saskatoon, Saskatchewan N.R.C. No. 2952 (1952).
10) A.C. Neish, *Can., J. Chemistry*, **29**, 552 (1951).
11) J. Cerbulis, *Anal. Chem.*, **27**, 1400 (1955).
12) A. Yoda, *J. Chem. Soc. Japan*, **73**, 18 (1952).
13) N.D. Cheronis and J.B. Entriken, Semimicro qualitative organic analysis 2nd Ed. 1957, p. 368.

high concentration of yeast extract.

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Studies on Osmophilic Yeasts*

Part XII. Characteristic Aspects on the Assimilation and Fermentation of Sugars by Osmophilic Yeasts in the Environment of a High Concentration of Sodium Chloride

By Hiroshi Ōnishi

Assimilation of galactose and maltose by *Saccharomyces rouxii*, which is a typical salt-tolerant yeast playing an important role in soy-brewing, was negligible or extremely poor in the medium containing 18% NaCl, although the assimilation in the ordinary medium was vigorous. The yeasts which were able to assimilate and ferment galactose, maltose and/or saccharose in the high-saline medium were limited to a few strains. From the studies on balances of fermentation products, it was revealed that ethanol yield based on sugar consumed in the 18% NaCl-medium was lower than that in the ordinary medium, suggesting that other fermentation products than ethanol should be accumulated. Analytical results of the fermented broths showed much polyalcohol production in the high-saline medium.

INTRODUCTION

Comparative studies on the metabolism of osmophilic yeasts in the ordinary medium and in the high-saline environment have been reported by the author[1~3].

Takahashi and Yukawa[4] and Ishimaru[5] presented the analytical data of the broths fermented by soy-yeasts, although they did not refer to the special behavior of yeasts in the high-saline medium.

In this paper will be described some characteristic assimilation and fermentation of sugars by various salt-tolerant yeasts in the saline condition with reference to the shift of metabolism as compared wtih the ordinary culture.

The studies on these characteristics of the salt-tolerant yeasts might be not only of great use in the manufacture of soy-sauce and miso paste, but also full of interest in terms of the taxonomy of osmophilic and halophilic micro-organisms.

EXPERIMENTAL AND RESULTS

[I] Influence of a High Concentration of Sodium Chloride on Sugar Assimilation by Salt-tolerant Yeasts.

Sixty-nine salt-tolerant yeast strains[6,7] of various genera and species were examined as for their sugar assimilation in the ordinary media and those containing 18% NaCl. The media employed were as follows: (1): KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCl_2 \cdot 2H_2O$ 0.01%, NaCl 0.01%, Bacto vitamin-free casamino acid 0.4%, Bacto yeast ext. 0.1%, either glucose, galactose, maltose or saccharose 3%. (2): (1)+NaCl 18%.

One loopful of the yeast taken from the fresh culture on koji extract agar was suspended in sterile saline. Attention was paid so as not to touch the agar

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1) H. Ōnishi, Bull. Agr. Chem. Soc. of Japan, **21**, 143 (1957).
2) H. Ōnishi, *ibid.*, **23**, 332 (1959).
3) H. Ōnishi, *ibid.*, **23**, 352 (1959).
4) T. Takahashi and M. Yukawa, *Memoirs of the College of Agr., Tokyo Imp. Univ.*, **5**, 227 (1915).
5) Y. Ishimaru, *J. Fermentation Technol.*, **13**, 295 (1935).
6) H. Ōnishi, Bull. Agr. Chem. Soc. of Japan, **21**, 151 (1957).
7) H. Ōnishi, *ibid.*, **24**, 386 (1960).

TABLE I. INFLUENCE OF A HIGH CONCENTRATION OF SODIUM CHLORIDE ON THE SUGAR ASSIMILATION BY SALT-TOLERANT YEASTS

Strains			Glucose		Galactose		Maltose		Saccharose	
			NaCl 0%	NaCl 18%	NaCl 0%	NaCl 18%	NaCl 0%	NaCl 18%	NaCl 0%	NaCl 18%
Sacch. rouxii	m	1	##	##	##	–	##	–	##	±
//	m	2	##	##	+	–	##	–	+	±
//	m	3	##	##	##	–	##	–	##	±
//	m	4	##	##	##	–	##	–	##	+
//	m	5	##	##	##	–	##	–	##	±
//	m	6	##	##	##	–	##	–	##	±
//	m	8	##	##	##	–	##	–	##	±
//	m	9	##	##	##	–	##	–	##	±
//	m	10	##	##	##	–	##	–	##	±
//	m	11	##	##	##	–	##	–	+	±
//	m	14	##	##	##	–	##	+	##	+
//	s	2	##	##	##	–	##	–	##	+
//	s	4	##	##	##	–	##	–	##	±
//	s	5	##	##	##	–	##	–	##	##
//	s	8	##	##	+	–	##	–	+	±
//	s	11	##	##	+	–	##	–	+	±
//	s	12	##	##	##	–	##	–	+	±
//	sal	1	##	##	##	–	##	–	+	±
//	sal	2	##	##	##	–	##	–	+	±
//	sal	3	##	##	##	–	##	–	+	±
//	sal	5	##	##	##	–	##	–	+	±
//	j	4	##	##	±	–	##	–	±	–
//	j	5	##	##	±	–	##	–	±	–
//	R	4	##	##	+	–	##	–	+	±
//	R	5	##	##	+	–	##	##	+	+
//	E	7	##	##	##	–	##	–	+	±
//	N	3	##	##	##	–	##	–	##	–
//	N	4	##	##	+	–	##	–	+	±
//	N	5	##	##	±	–	##	–	±	±
//	N	6	##	##	##	–	##	–	+	±
//	N	8	##	##	##	–	##	–	+	±
//	N	9	##	##	+	–	##	–	±	±
//	N	10	##	##	##	–			+	±
//	N	11	##	##	±	–			±	±
//	N	11a	##	##	##	–			+	±
//	N	15	##	##	±	–			±	±
//	N	13a	##	##	##	–			±	±
//	N	18	##	##	##	–			##	+
//	N	28	##	##	+	–			+	±
//	A	34	##	##	+	–	##	–	+	±
Sacch. rouxii var.	*halomembranis*	sal 4	##	##	##	–	##	–	##	##
//		sal 6	##	##	##	–	##	–	##	±
//		j 3	##	##	##	##	##	##	##	##
//		j 6	##	##	##	+	##	##	##	##
//		N 16	##	##	##	##	##	–	+	+

TABLE I. (Continued)

Strains		Glucose		Galactose		Maltose		Saccharose	
		NaCl 0%	NaCl 18%	NaCl 0%	NaCl 18%	NaCl 0%	NaCl 18%	NaCl 0%	NaCl 18%
Sacch. rouxii var. *halomembrains*	N 22	++++	++++	++++		++++		++	++++
//	N 26	++++	++++	++++		++++		++++	±
//	A 31	++++	++++	++++	−	++++	++++	++++	++++
Sacch. acidifaciens	s 9	++++	++++	+	−	++++	−	+	±
Sacch. acidifaciens var. *halomembranis*	R 6	++++	++++	++++	−	+	−	++++	++++
Torulopsis halophilus	N 24	++++	++++	++++	+	+	++++	++++	+
Torulopsis nodaensis	N 21	++++	++++	++++	++++	−	−	+	+
Torulopsis famata	E 29a	++++	++++	++++	++++	++++	±	++++	++++
Torulopsis versatilis	F 5	++++	++++	++++	+	++++	++++	++++	++++
//	F 7	++++	++++	++++	++++	++++	++++	++++	++++
Torulpsis spaerica	F 8	++++	++++	++++	±	±	−	++++	++++
//	F 28	++++	++++	++++	++++	+	±	++++	++++
Torulopsis sake	F 11	++++	++++	++++	+	++++	++++	++++	++++
Torulopsis etchellsii	A 23	++++	++++	++++	−	++++	++++	++++	++++
Pichia farinosa	A 6	++++	++++	++++	++++	++++	++++	++++	++++
Candida polymorpha	Ek	++++	++++	++++	++++	++++	++++	++++	++++
Trichosporon behrendii	E 3a	++++	++++	++++	++++	++++	±	++++	++++
Hansenula subpelliculosa	H 79	++++	++++	++++	±	++++	−	++++	++++
Debaryomyces hansenii	H 70	++++	++++	++++	++++	++++	++++	++++	++++

layer. One drop of this suspension was inoculated to each medium. After 15 and 30 days' incubation at 30°C, the extent of assimilation was judged from the growth of cells. The results are shown in Table I.

As expected, all the strains tested were able to assimilate glucose well in both media, ordinary and 18% NaCl. When galactose or maltose was used as a sole carbon source, *Sacch. rouxii* and *Sacch. rouxii* var. *holomembranis* showed good growth in the ordinary medium, whereas negligible or extremely poor growth was observed in the 18% NaCl-medium even after incubation for 30 days. Most of *Pichia* and *Torulopsis* could utilize galactose and maltose even in the 18% NaCl medium.

[II] Influence of a High Concentration of Sodium Chloride on Sugar Fermentabilities by the Salt-tolerant Yeasts.

From the experiment [I], it was found that assimilations of sugars other than glucose by the salt-tolerant yeasts were suppressed by the presence of 18% NaCl. Sugar fermentabilities were then examined on the selected strains which showed relatively good assimilation of the miscellaneous sugars even in the 18% NaCl medium.

First, semiquantitative fermentation test employing Durham tube was carried out. As shown in Table II, the fermentation of sugars other than glucose in the 18% NaCl medium was poor.

Second, the time course of fermentation was examined quantitatively. Eighty ml of each of the media (the same as those in [I] except 5% sugars) were placed in a 150 ml Erlenmeyer flask, sterilized, inoculated with an organism and incubated at 30°C setting alwood tube. Evolution of CO_2 was gravimetrically estimated from the decrease in the total weight (Fig. 1). The salt-tolerant yeasts which were more or less able to ferment galactose, maltose or saccharose in the high-saline medium, were limited to few strains as follows:

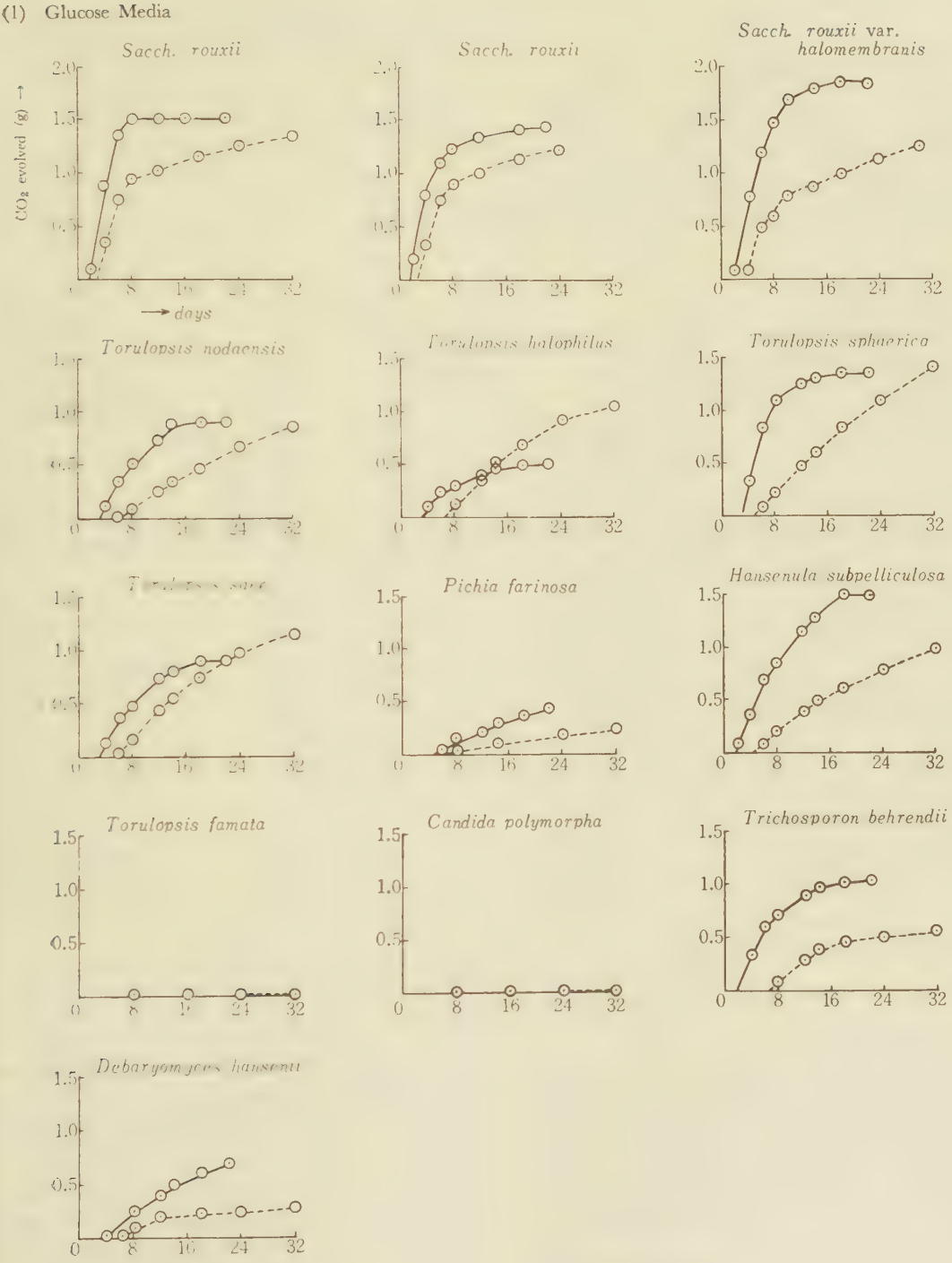
(1) Yeasts which ferment galactose.....*Torulopsis nodaensis* and *Torulopsis sake*.

(2) Yeasts which ferment maltose.....*Torulopsis versatilis*, *Torulopsis etchellsii* and *Torulopsis sake*.

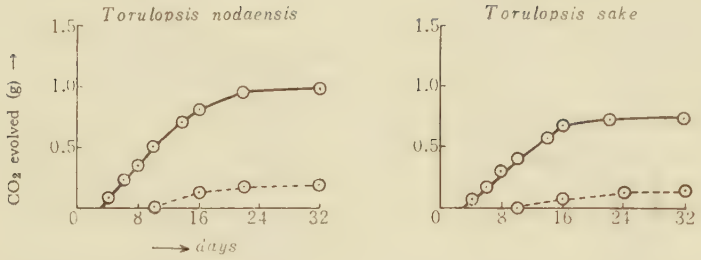
(3) Yeasts which ferment saccharose.....*Hansenula subpelliculosa* and *Torulopsis sphaerica*.

Sacch. rouxii, which plays an important role in soy-sauce brewing, could ferment glucose and maltose in the ordinary medium. In the presence of 18% NaCl, however, the yeast fermented only glucose, but not maltose. The time courses of fermentation by *Sacch.*

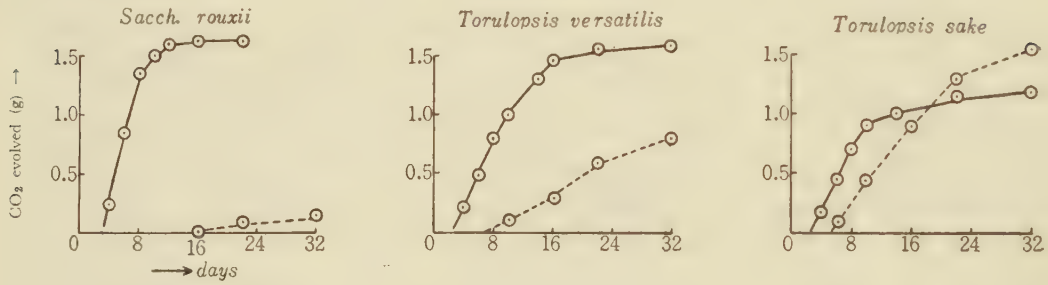
(1) Glucose Media



(2) Galactose Media



(3) Maltose Media



(4) Saccharose Media

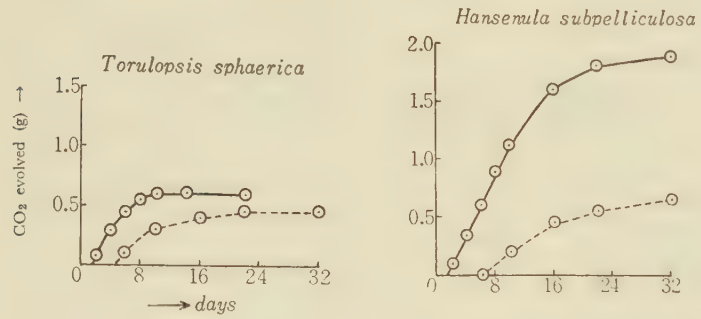


FIG. 5. Influence of a High Concentration of Sodium Chloride on the Fermentation of Sugars by Salt-tolerant Yeasts.
Solid line: NaCl 0% Broken lsne: NaCl 18%

rouxii in the four types of media containing mixtures of glucose and maltose at various ratios are illustrated in Fig. 2. Similar curves showing the absence of effects of the mixing ratios of glucose and maltose were obtained in the media free of NaCl. On the contrary, four curves in the 18% NaCl-media were quite different; the fermentation proceeded in proportion to the amounts of glucose added.

[III] Influence of a High Concentration of Sodium Chloride on Balances of the Fermentation Products by the Salt-tolerant Yeasts.

Highly salt-tolerant 18 strains of various genera and species, which were found in soy-sauce mashes and miso-paste, were employed.

The media used are as follows. (1) Glucose 10%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCl_2 \cdot 2H_2O$ 0.01%, NaCl 0.01%, Bacto vitamin-free casamino acid 0.4%, and Bacto yeast ext. 0.1%. (2): (1) +NaCl 18%.

TABLE II. INFLUENCE OF A HIGH CONCENTRATION OF SODIUM CHLORIDE ON THE SUGAR FERMENTABILITY BY SALT-TOLERANT YEASTS

Strain		NaCl 0%	NaCl 18%
(1) Maltose			
Sacch. rouxii	m 14	##	+
//	R 5	##	+
Sacch. rouxii var. *halomembranis*	sal 4	##	−
//	j 3	##	−
//	j 6	##	−
//	N 16	##	−
//	N 22	##	±
//	A 31	##	−
Torulopsis sake	F 11	##	##
Torulopsis versatilis	F 5	##	+
//	F 7	##	##
Torulopsis etchellsii	A 23	##	##
(2) Galactose			
Torulopsis nodaensis	N 21	##	+
Torulopsis versatilis	F 5	##	−
//	F 7	##	−
Torulopsis sake	F 11	##	+
Torulopsis sphaerica	F 8	##	−
//	F 28	##	−
Trichosporon behrendii	E 3a	##	−
Debaryomyces hansenii	H 70	##	−
Pichia farinosa	A 6	##	−
(3) Saccharose			
Torulopsis versatilis	F 5	##	−
//	F 7	##	−
Torulopsis sake	F 11	##	−
Torulopsis sphaerica	F 8	##	+
//	F 28	##	+
Hansenula subpelliculosa	H 79	##	##

For stationary culture, 100 ml each of one of these media were placed in a 150 ml Erlenmyer flask, sterilized, inoculated and incubated at 30°C. Shaking culture was carried out with 80 ml of these media placed in 500 ml shaking flasks. The flasks were shaken on a reciprocal shaker operating at 120 r.p.m. with a stroke of 75 mm at 30°C.

The fermentation broths after removal of yeast cells by filtration, were analysed according to the following methods.

Glucose: The Fehling-Lehmann Schoorl method.

Ethanol: After distillation in neutral, the oxidation method by dichromate was applied.

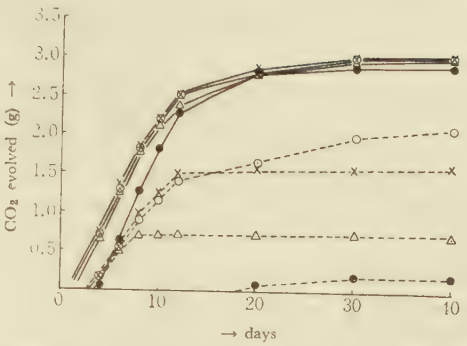


FIG. 2. Influence of a High Concentration of Sodium Chloride on the Fermentation of *Sacch. rouxii* in the Media Containing Glucose and Maltose at Various Ratio.

⊙: glucose : maltose=8 : 0 ×: glucose : maltose=4 : 4
△: glucose : maltose=2 : 6 ●: glucose : maltose=0 : 8
solid line : NaCl 0% broken line : NaCl 18%

pH: pH test paper (Toyo Roshi Kaisha).

Total acid: Ether extracts from the broth acidified with H_2SO_4, were titrated with N/20 NaOH.

Polyalcohol: The method of Neish[8,9] was used, i.e. the polyalcohols were oxidized with periodic acid and formaldehyde formed was determined colorimetrically with a chromotoripc reagent. The values obtained were corrected for glucose by running a standard glucose sample in parallel with the fermentation solutions.

2-3 Butanediol: A method was Desnuelle and Naudet's[10] as described by Neish[8], i.e. the oxidation with periodic acid followed by colorimetric determination of acetaldehyde formed with piperazine and nitroprusside solution. A correction was made for acetoin present.

Acetoin: The method of Westerfeld[11] as described by Neish[8], i.e. the colorimetric determination with creatine and α-napthol.

Sodium chloride did not interfere in the determination of polyalcohol and acetoin, but interfered in the determination of 2,3-butanodiol. In the latter case, a correction curve was prepared for the sample containing NaCl.

Analytical results of the fermented broths are given

8) A.C. Neish, Analytical methods for bacterial fermentations 2nd rev. Natl. Research Council. Can., Prairie Regional Lab., Sask. N.R.C. No. 2952 (1952).
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TABLE III. INFLUENCE OF A HIGH CONCENTRATION OF SODIUM CHLORIDE ON FERMENTATION PRODUCTS BY SALT-TOLERANT YEASTS. (1) STATIONARY CULTURE

Strains	Glucose consumed %	Ethanol formed %	Ethanol yield on glucose consumed*	pH	Total acid % (as succinate)	Polyalcohol formed % (as glycerol)	Polyalcohol yield on glucose consumed (as glycerol)	2, 3-Butanediol formed %	Acetoin formed %
(a) NaCl-free medium (15 days' cultivation)									
Sacch. rouxii N 28	5.25	1.82	67.8%	3.8	0.069	0.409	7.8%	0.033	0.009
// E 7	6.20	2.24	70.6	3.4	0.099	0.504	8.1	0.027	0.003
Sacch. rouxii var. *halomembranis* A 31	7.62	3.01	77.3	3.4	0.111	0.345	4.5	0.025	0.002
Sacch. rouxii var. *halomembranis* sal 4	6.41	2.49	75.9	3.4	0.111	0.290	4.5	0.023	0.003
Sacch. acidifaciens	6.88	2.25	64.0	3.6	0.078	1.128	16.4	0.030	0.010
Sacch. acidifaciens var. *halomembranis*	8.55	3.19	73.0	3.4	0.114	0.762	8.9	0.025	0.002
Torulopsis nodaensis	4.81	1.68	68.3	3.2	0.141	0.544	11.3	0.011	0.001
Torulopsis halophilus	3.52	1.18	65.3	3.4	0.075	0.363	10.2	0.016	0.001
Torulopsis versatilis	2.52	0.71	55.1	3.6	0.099	0.387	15.3	0.007	0.001
Torulopsis sphaerica	4.53	1.34	57.9	2.4	0.159	1.014	22.3	0.014	0.002
Torulopsis sake	3.91	1.34	67.1	3.4	0.105	0.433	11.0	0.015	0.001
Torulopsis etchellsii	3.06	1.07	68.3	3.4	0.069	0.243	7.9	0.007	0
Torulopsis famata	1.29	0.06	39.3	4.2	0.027	0.096	7.4	0.003	0.001
Pichia farinosa	4.99	1.09	42.7	3.8	0.087	0.777	15.5	0.008	0
Candida polymorpha	1.15	0.19	32.3	4.8	0	0.172	14.9	0.003	0.001
Trichosporon behrendii	3.15	1.09	67.2	3.6	0.069	0.229	7.2	0.004	0
Hansenula subpelliculosa	5.21	2.00	75.1	3.8	0.069	0.325	6.2	0.007	0
Debaryomyces hansenii	3.08	0.62	39.3	4.0	0.045	0.611	19.8	0.004	0
(b) NaCl 18% medium (18 days' cultivation)									
Sacch. rouxii N 28	5.51	1.63	57.3%	3.4	0.075	0.573	10.3%	0.039	0.003
// E 7	5.10	1.34	51.4	3.4	0.078	0.759	14.8	0.036	0.003
Sacch. rouxii var. *halomembranis* A 31	6.03	1.92	62.2	3.2	0.075	0.509	8.4	0.029	0.001
Sacch. rouxii var. *halomembranis* sal 4	4.82	1.38	56.0	3.2	0.099	0.702	14.5	0.024	0.001
Sacch. acidifaciens	5.36	1.53	55.8	3.4	0.090	0.854	16.0	0.025	0.003
Sacch. acidifaciens var. *halomembranis*	3.92	1.15	57.4	3.4	0.096	0.683	17.4	0.023	0.001
Torulopis nodaensis	3.64	1.11	59.6	3.2	0.093	0.366	10.0	0.010	0.001
Torulopsis halophilus	4.27	1.39	63.6	3.2	0.078	0.300	7.0	0.010	0.001
Torulopsis versatilis	2.60	0.69	51.9	3.4	0.069	0.419	16.1	0.017	0.002
Torulopsis sphaerica	4.58	1.56	66.6	3.4	0.069	0.308	6.7	0.013	0.001
Torulopsis sake	4.16	1.34	63.0	3.4	0.063	0.372	8.9	0.021	0.001
Torulopsis etchellsii	4.03	1.29	62.6	3.2	0.075	0.256	6.3	0.001	0
Torulopsis famata	0.88	0.04	8.9	4.0	0.006	0.130	14.7	0	0
Pichia farinosa	4.44	0.07	3.1	3.2	0.063	1.162	26.1	0.001	0
Candida polymorpha	1.14	0.13	22.3	4.0	0.003	0.142	12.4	0.002	0.001
Trichosporon behrendii	1.92	0.19	19.3	4.0	0.003	0.238	12.3	0.002	0
Hansenula subpelliculosa	5.10	1.59	61.0	3.4	0.069	0.571	11.2	0.031	0.004
Debaryomyces hansenii	3.75	0.06	3.1	3.6	0.039	0.991	26.4	0.001	0

*This valuc was conventionally calculated from Gay-Lussac formula of ethanol fermentation.

TABLE IV. INFLUENCE OF A HIGH CONCENTRATION OF SODIUM CHLORIDE ON FERMENTATION PRODUCTS BY SALT-TOLERANT YEASTS. (2) SHAKING CULTURE

Strains		Glucose consumed %	Ethanol formed %	Ethanol yield on glucose consumed*	pH	Total acid % (as succinate)	Polyalcohol formed % (as glycerol)	Polyalcohol yield on glucose consumed (as glycerol)	2, 3-Butanediol formed %	Acetoin formed %
(a) NaCl-free medium (6 days' cultivation)										
Sacch. rouxii	N 28	9.02	1.12	24.3%	3.8	0.009	1.282	14.2%	0.167	0.064
"	E 7	9.18	0.07	1.5	3.4	0.024	1.995	21.7	0.031	0.003
Sacch. rouxii var. *halomembranis*	A 31	8.50	0.28	6.4	2.6	0.054	1.664	19.5	0.028	0.006
Sacch. rouxii var. *halomembranis*	sal 4	7.06	1.60	44.3	3.4	0.033	0.899	12.7	0.158	0.080
Sacch. acidifaciens		6.42	1.23	27.4	3.4	0.078	1.108	17.2	0.072	0.038
Sacch. acidifaciens var. *halomembranis*		9.26	0.04	0.7	3.4	0.051	2.587	25.2	0.016	0.003
Torulopsis nodaensis		1.65	0.05	5.9	2.2	0.210	0.055	3.3	0.008	0.001
Torulopsis halophilus		1.76	0.05	5.6	2.6	0.048	0.056	3.1	0.003	0
Torulopsis versatilis		2.41	0.03	2.4	2.2	0.099	0.849	35.2	0.005	0
Torulopsis sphaerica		2.07	0.01	1.0	2.0	0.189	0.544	26.2	0.007	0
Torulopsis sake		2.19	0.07	6.3	2.2	0.159	0.400	18.2	0.008	0
Torulopsis etchellsii		5.02	0.02	0.8	2.8	0.117	0.067	1.3	0.007	0
Torulopsis famata		7.24	0.02	0.5	4.0	0	1.657	22.9	0.005	0
Pichia farinosa		9.18	0.03	0.6	2.6	0.009	1.695	18.4	0.001	0
Candida palymorpha		8.23	0.02	0.5	4.0	0	1.952	23.7	0.002	0
Trichosporon behrendii		9.21	0.05	1.0	4.0	0	0.246	2.6	0.008	0.001
Hansenuta subpelliculosa		9.11	0.18	3.8	2.4	0.129	0.541	6.0	0.043	0.028
Debaryomyces hansenii		4.15	0.67	21.5	3.4	0.054	0.569	13.7	0.011	0.001
(b) NaCl 18% medium (10 days' cultivation)										
Sacch. rouxii	N 28	7.66	0.07	1.8%	3.2	0.057	2.843	37.1%	0.025	0.006
"	E 7	7.83	0.08	2.0	3.0	0.030	2.248	28.7	0.028	0.002
Sacch. rouxii var. *halomembranis*	A 31	6.43	0.03	0.9	3.0	0.057	1.567	23.5	0.025	0.001
Sacch. rouxii var. *halomembramis*	sal 4	1.25	0.22	34.4	3.8	0.009	0.167	13.3	0.010	0.008
Sacch. acidifaciens		3.89	0.37	18.6	3.4	0.039	1.344	34.5	0.024	0.008
Sacch. acidifaciens var. *halomembranis*		1.79	0.21	22.4	3.8	0.027	0.227	12.7	0.007	0.002
Torulopsis nodaensis		1.53	0	0	2.0	0.093	0.102	6.6	0	0
Torulopsis halophilus		2.30	0.01	0	3.6	0.003	0.312	13.5	0	0
Torulopsis versatilis		1.37	0.23	32.8	2.6	0.069	0.171	12.4	0.005	0.002
Torulopsis sphaerica		1.05	0.15	27.9	2.6	0.066	0.146	13.9	0.005	0.001
Torulopsis sake		1.05	0.11	20.5	2.6	0.166	0.086	8.2	0	0
Torulopsis etchellsii		2.05	0.03	3.0	3.6	0	0.037	1.8	0.002	0
Torulopsis famata		6.93	0	0	4.2	0	0.058	0.8	0	0
Pichia farinosa		7.96	0.01	0	3.6	0.012	1.228	15.4	0.001	0
Candida polymorpha		7.28	0	0	4.2	0	0	0	0	0
Trichosporon behrendii		3.76	0	0	4.2	0	0.237	6.3	0.001	0
Hansenula subpelliculosa		5.72	0	0	2.6	0.033	1.667	29.1	0	0
Deharyomyces hansenii		3.39	0.02	1.0	3.6	0.009	1.131	33.3	0.007	0.001

* This value was conventionally calculated from Gay-Lussac formula of ethanol fermentation.

in Table III and IV. It was revealed that the amount of ethanol produced in the 18% NaCl-medium was much less than that expected from the amount of glucose consumed. It might be that some alternative products were formed in the high-saline medium From the analyses of acids, polyalcohols, 2,3-butanediol and acetoin, it was found that much polyalcohol was produced in the high-saline medium. Such polyalcohol production had been previously reported by the author in detail[12~14].

DISCUSSION

Sugar assimilability and fermentability are the most important criteria in the taxonomy of yeasts. Present observation that these properties of the salt-tolerant yeasts can be drastically altered by changing the concentration of NaCl in the medium, should be noteworthy not only in the taxonomy of the osmophilic and halophilic microorganisms, but also in the industrial utilization of these microorganisms.

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Vitamin and Amino Acid Requirements of *Pediococcus soyae* and *Pediococcus acidilactici* Kitahara's Strain *

Part IX. Studies on the Activities of Bacteria in Soy Sauce Brewing

By Kenji Sakaguchi

Nutritional requirements of three strains of *Ped. soyae*[1,2] one strain of *Ped. acidilactici* (Kitahara's strain) were determined from a complete synthetic medium. Upon inspection by the single omission method, *Ped. soyae* required glycine-betaine[3], uracil, riboflavine, pyridoxine or pyridoxal, pantothenic acid, nicotinic acid, leucovorin, biotin, glutamic acid, arginine, histidine, isoleucine, leucine, phenyl alanine, valine, tryptophane, methionine, cystine and serine with exception of the P-factor[4]. In addition to the above mentioned nutrients, a representative strain of the organism required xanthine, folic acid, thiamine, aspartic acid and threonine for the minimum synthetic medium. *Ped. acidilactici* Kitahara's strain required riboflavine, pyridoxine, pantothenic acid, nicotinic acid, biotin and all components of the basal medium consisting of amino acids except methionine, but it did not require leucovorin, glycine-betaine and organic bases. Nutritional requirements of *Ped. pentosaceus*, Kitahara's strain[5,6] was proved to be quite identical with *Ped. cerevisiae* Pederson[7~9] (=*Ped. pentosaceus* Mees[12]).

The genus *Pediococcus* was first attached to the family *Lactobacillaceae* in the 7th edition of Bergey's Manual[7] as the result of laborious works of Pederson[7~9] and others[10,11]. However, in this genus only two species are included, namely *Ped. cerevisiae* and *Ped. acidilactici*, and nothing concerning salt-tolerant and dextrorotatory lactic acid forming pediococci is described[1,2,5,12]. Moreover, Nakagawa and Kitahara[5] concluded that type cultures of the so-called *Ped. cerevisiae* classified by Pederson is not a true beer-sarcina known as *Pediococcus cerevisiae* Balcke, it should be included in *Ped. pentosaceus* Mees[5,12].

The nutritional requirements of *Ped. pentosaceus* were first studied by Sauberlich and Baumann[13] with a strain of this genus formerly known as *Leuconostoc citrovorum* ATCC 8081[8] and specific requirements for leucovorin (folinic acid) were elucidated. Thereafter, Jensen and Seeley have reported on the minimum synthetic medium for 34

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strains of this species, and concluded that requirements of these strains for vitamins, bases and amino acids were quite uniform.

As for the thermotolerant pediococcus *Ped. acidilactici* Lindner, a strain of the species[5], *Leuconostoc mesenteroides* P-60 was thoroughly investigated for the purpose of microbiological bioassay by Snell[14~16], Dunn[17] and Steele[18].

Recently, Nakagawa and Kitahara have offered a probable proposal on the general classification of the genus *Pediococcus,* and nutritional requirements of the member species were also studied. It was unfortunate that Difco's vitamin-free casamino acids were used as the amino acid source, as this acid hydrolysed milk casein is not actually vitamin-free, but contamination of unsaturated fatty acids[20], an unknown growth factor for *Ped. soyae* (P-factor)[4,21] and other substances as diketopyperazines or amines have been reported or suspected. On the whole it may be said that, Nakagawa and Kitahara's report, is, in most cases convincible as a general classification of the genus, but its nutritional descritpion is imperfect especially in the case of *Ped. soyae.* Naturally, amino acid requirements of the genus were not referred in their report.

The present paper deals with the nutritional requirements of *Ped. soyae* inspected by the single omission method and by the minimum synthetic medium which comprises glycine-betaine[3] as a specific nutrient for the organism. Furthermore, the vitamin and amino acid requirements of *Ped. pentosaceus* Kitahara's strain are studied by the single omission method from the complete synthetic medium and the results obtained are offered for general studies of this genus and for subsequent studies of this series.

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MIETHODS

Basal medium. The composition of the basal medium employed was identical with that listed in the previous report[3]. That is, the standard synthetic medium for lactic acid bacteria was supplemented with glycine-beatine hydrochloride, 30 mg/l, choline hydrochloride, 2 mg/l, inositol, 2 mg/l, and leucovorin (folinic acid) 10 μg/l, but pyridoxal was omitted here.

Cultures. Three strains of *Ped. soyae,* d 2, d 8 and dl 2 were selected from the author's isolates because of their heterogenity in sugar fermentabilities or optical rotation of produced lactic acid[19].

The thermotolerant strain *Ped. acidilactici* Kitahara's strain originally isolated from a corn mash by K. Kitahara[6] in 1940, and classified at that time as a strain of *Ped. lindneri* Henneberg. At the same time *Ped. pentosaceus* Kitahara's strain was isolated from another corn mash and was assigned its former name *Ped. hennebergi* Sollied. Recently, taxonomical identification of the two strains were investigated again by Nakagawa and Kitahara[5] and were given the name of new species.

Assay method. Procedures were identical with that described in the former reports. After four days' subculture in the case of *Ped. soyae,* and after two days' in the case of *Ped. acidilactici* or *Ped. pentosaecus,* the cells were centrifuged, washed four times with 0.9% potassium chloride solution, and diluted to give a final optical density of approx. 0.07, at 660 mμ. Five-ml basal medium omitted with each one of the nutrients were dispensed in cotton-plugged test tubes, autoclaved for fifteen minutes at 120°C, cooled, incubated with one drop per tube of the cell suspension described above and incubated at 30°C. After incubation for an appropriate period, growth was estimated photometrically at 660 mμ, or the acid produced was titrated N/20 sodium hydroxide solution. Quadruplicate tests for one sample were run as usual and growth was assayed periodically.

RESULTS AND DISCUSSION

I Vitamin and Base Requirements of *Ped. soyae*

Three strains of *Ped. soyae* d 2, d 8 and dl 2

TABLE I. VITAMIN AND BASE REQUIREMENTS OF *Ped. soyae*

Eliminated vitamins	d 2 O.D. 5 days	d 2 O.D. 8 days	d 2 Acid produced (N/20 NaOH ml)	d 8 O.D. 5 days	d 8 O.D. 8 days	d 8 Acid produced (N/20 NaOH ml)	dl 2 O.D. 5 days	dl 2 O.D. 8 days	dl 2 Acid produced (N/20 NaOH ml)
none	0.40	0.37	1.60	0.33	0.44	2.14	0.41	0.37	1.75
adenine sulphate	0.40	0.39	1.79	0.35	0.43	2.18	0.39	0.43	2.15
guanine·HCl	0.42	0.43	1.60	0.33	0.43	2.21	0.43	0.45	1.95
uracil	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.25	0.73
xanthine	0.41	0.43	1.68	0.31	0.37	1.82	0.45	0.47	2.00
riboflavine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
thiamine·HCl	0.38	0.39	1.55	0.33	0.44	2.26	0.38	0.42	1.84
PABA	0.39	0.40	1.56	0.33	0.45	2.25	0.40	0.43	1.92
pyriodoxine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ca-*dl*-pantothenate	0.01	0.02	0.40	0.00	0.00	0.00	0.00	0.00	0.00
nicotinic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
biotin acid	0.14	0.12	0.84	0.07	0.04	0.23	0.13	0.13	0.78
folic acid	0.40	0.41	1.55	0.32	0.41	2.11	0.43	0.45	1.93
inositol	0.33	0.37	1.50	0.30	0.37	1.85	0.40	0.43	2.00
leucovorin	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
choline·HCl	0.35	0.39	1.61	0.39	0.42	2.25	0.39	0.43	1.97
glycine-betaine·HCl	0.20	0.28	1.20	0.07	0.16	1.05	0.15	0.24	1.04

which had different characters to each other in sugar fermentability and optical rotation of produced lactic acid were selected and inoculated into the media which were eliminated by one of each vitamin or base from the basal snythetic medium. Growth was estimated turbidimetrically after five and eight days' and acidmetrically after eight days' culture. The results shown in Table I indicate that all three strains essentially require uracial, riboflavine, pantothenic acid, nicotinic acid, leucovorin, pyridoxine, while stimulatory growth effects are observed with biotin and glycine-betaine. However, strain d 2 required pantothenic acid and strain dl 2 required uracil only stimulatorily. In Part III of this series it was reported that *Ped soyae does* not require leucovorin. This was proved to be errorneous in this present experiment. The error is thought to have occurred from the inevitable use of incomplete synthetic media at that time.

When the vitamin and base requirements are compared between *Ped. soyae* and salt sensitive *Ped. pentosaceus* Mees[5,10] (=*Ped. cerevisiae* Pederson[7~9]. Its nutritional study has been made by Jensen and Seeley[11].) general tendencies such as the necessity for leucovorin agreed well but stimulatory requirements for uracil, riboflaivne and pyridoxine of *Ped. pentosaceus* changed more strictly to essential requirements in the case of *Ped. soyae*. This phenomenon will be infered later in a subsequent report.

As stated later, *Ped. acidilactici,* also have the same tendency in spite that they do not require leucovorin and uracil.

II Amino Acid Requirements of *Ped. soyae*

Although vitamin requirements of *Ped. soyae* had a tendency resembling other salt-sensitive pediococci, their amino acid requirements were quite different. From experiments later described and other reports[12~16] it became clear that the salt tolerant pediococci needs almost all of the amino acids for

Table II. Amino Acid Requirements of *Ped. soyae*

Eliminated amino acid	d 2 O.D. 5 days	d 2 O.D. 8 days	d 2 Acid produced (N/20 NaOH ml)	d 8 O.D. 5 days	d 8 O.D. 8 days	d 8 Acid produced (N/20 NaOH ml)	dl 2 O.D. 5 days	dl 2 O.D. 8 days	dl 2 Acid produced (N/20 NaOH ml)
none	0.40	0.37	1.60	0.33	0.44	2.14	0.41	0.37	1.75
DL alanine	0.37	0.36	1.64	0.27	0.34	2.10	0.42	0.43	2.00
DL aspartic acid	0.37	0.38	1.50	0.26	0.40	2.13	0.34	0.42	1.87
L glutamic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L arginine·HCl	0.01	0.03	0.46	0.01	0.01	0.28	0.01	0.01	0.40
L lysine·HCl	0.34	0.31	1.41	0.25	0.32	1.75	0.28	0.34	1.70
L histidine·HCl	0.00	0.00	0.22	0.00	0.00	0.00	0.00	0.00	0.19
DL isoleucine	0.00	0.00	0.25	0.00	0.00	0.00	0.00	0.00	0.00
DL methionine	0.18	0.20	1.14	0.19	0.24	1.49	0.19	0.20	1.16
DL phenylalanine	0.00	0.00	0.00	0.31	0.41	2.00	0.29	0.35	1.70
L proline	0.36	0.35	1.55	0.32	0.37	1.95	0.30	0.38	1.94
DL threonine	0.30	0.30	1.40	0.20	0.30	1.80	0.28	0.32	1.70
L tyrosine	0.37	0.35	1.63	0.25	0.34	1.75	0.30	0.35	1.79
DL valine	0.00	0.00	0.25	0.00	0.00	0.00	0.00	0.00	0.11
DL tryptophane	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
L cystine	0.19	0.21	1.25	0.00	0.22	1.45	0.11	0.16	1.25
DL serine	0.22	0.23	1.10	0.00	0.20	1.40	0.16	0.20	1.20
Glycine	0.36	0.34	1.51	0.16	0.38	1.82	0.29	0.33	1.75
L leucine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

growth, but salt tolerant pediococcus *Ped. soyae* requires only glutamic acid, arginine, histidine, isoleucine, phenyl alanine, valine, tryptophane and leucine essentially, and methionine, cystine and serine stimulatorilly. The organism does not require alanine, aspartic acid, lysine, proline, threonine, tyrosine and glycine in case of the single omission experiment (Table II). The requirement for phenyl alanine is only exhibited by strain d 2.

It is noteworthy that salt tolerant pediococci were proved to more complex vitamin while more simple amino acids than salt sensitive pediococci are required.

III The Minimum Synthetic Medium for *Ped. soyae* Strain d 2

In order to exhibit the minimum synthetic medium for *Ped. soyae,* a medium composed exclusively of required vitamins, bases and amino acids manifested by the above described two experiments were prepared with the addition of glucose and salts (primary medium; the constituents were, uracil, riboflavine, pantothenic acid, nicotinic acid, leucovorin, pyridoxine, biotin, glycine-betaine, glutamic acid, arginine, histidine, isoleucine, phenylalanine, valine, tryptophane, leucine, methionine, cystine, serine, glucose and salts.). However, on this medium, no growth of *Ped. soyae* strain d 2 was observed. After repeated experiments of supplementing other nutrients, normal grotwh of the strain d 2 was exhibited

Table III. Growth of *Ped. soyae* d2 on the Minimum Synthetic Medium

	O.D.	Acid produced (N/20 NaOH, ml)
Basal mecium	0.299	3.40
Medium composed of essential and stimulatory nutrients on Table I and II	0.00	0.00
+ xanthine, folic acid, thiamine, aspartic acid and threonine	0.267	2.84

Assays were made after five days' culture.

on the medium supplemented with xanthine, folic acid, thiamine, aspartic acid and threonine on the primary medium (Table III). It is thus considered that these supplemented vitamins and amino acids can be synthesized in the cell of *Ped. soyae* when one of each nutrient is singly eliminated from the basal complete medium though they can not be synthesized from nutrients constructing the primary medium. Therefore, it is presumed that biosynthesis of the five supplemented nutrients is accomplished by the metabolic action of one or more of these nutrients.

IV Vitamin and Amino Acid Requirements of *Ped. acidilactici* Kitahara's strain and *Ped. pentosaceus* Kitahara's strain

Ped. acidilactici Kitahara's strain and *Ped. pentosaceus* Kitahara's strain were originally isolated from corn mashes incubated at 40°C by Kitahara[5,6] in 1940, and then identified as *Ped. lindneri* Henneberg and *Ped. hennebergi* Sollied, respectively. Taxonomical studies were recently resumed and the new species names were bestowed.

On examining the nutritional requirements of the two strains, it was observed that the requirements of *Ped. pentosaceus* Kitahara's strain had identical vitamin, base and amino acid requirements with *Ped. cerevisiae* Pederson (=*Ped. pentosaceus* Mees[5,10]) which was determined by Jensen and Seeley in 1954[11] (Tables IV and V). It is a marvelous fact that only morphologically resembling strains isolated from such apart places and in different ages have such uniform nutritional requirements. The requirements were as follows; purines and pyrimidines not required, leucovorin, pantothenic acid, nicotinic acid, biotin and all amino acids required. Of these requirements, biotin, lysine, methionine and serine were stimulatory. The identification of this strain *Ped. pentosaceus* was thus also confirmed from the view point of nutritional studies.

Vitamins necessary for *Ped. acidilactici* Kitahara's strain were, riboflavine, pyridoxine, pantothenic acid, nicotinic acid and biotin, while purines, pyrimidines or especially, leuco-

Table IV. Vitamin and Base Requirements of *Ped. pentosaceus* Kitahara's Strain and *Ped. acidilactici* Kitahara's Strain

Eliminated vitamins	*Ped pentosaceus* O.D.	*Ped pentosaceus* Acid produced (N/20 NaOH ml)	*Ped. acidilactici* O.D.	*Ped. acidilactici* Acid produced (N/20 NaOH ml)
none	1.22	6.47	0.28	1.55
adenine sulphate	1.24	6.73	0.27	1.15
guanine·HCl	1.20	6.65	0.29	1.35
uracil	1.26	6.78	0.27	1.40
xanthine	1.24	6.40	0.31	1.65
riboflavine	1.26	6.72	0.00	0.20
thiamine·HCl	1.24	6.43	0.24	1.26
PABA	1.12	6.00	0.31	1.66
pyridoxine	1.18	6.49	0.00	0.00
Ca-*dl*-pantothenate	0.05	0.68	0.05	0.40
nicotinic acid	0.05	0.00	0.00	0.00
biotin	0.07	0.50	0.04	0.30
folic acid	1.20	6.40	0.29	1.48
inositol	1.24	6.61	0.29	1.55
leucovoin	0.05	0.45	0.34	1.30
choline·HCl	1.24	6.60	0.37	1.94
betaine·HCl	1.20	6.40	0.36	2.05

Assays were made after three days' culture.

Table V. Amino Acid Requirements of *Ped. pentosaceus* Kitahara's Strain and *Ped. acidilactici* Kitahara's Strain

Eliminated amino acids	*Ped. pentosaceus* O.D.	*Ped. pentosaceus* Acid produced (N/20 NaOH ml)	*Ped. acidilatici* O.D.	*Ped. acidilatici* Acid produced (N/20 NaOH ml)
none	1.22	6.47	0.28	1.55
DL alanine	0.00	0.00	0.05	0.26
DL aspartic acid	0.01	0.00	0.00	0.00
L glutamic acid	0.00	0.00	0.00	0.00
L arginine·HCl	0.02	0.21	0.00	0.30
L lysine·HCl	0.67	4.23	0.00	0.00
L histidine·HCl	0.00	0.00	0.00	0.00
DL isoleucine	0.00	0.00	0.00	0.00
DL methionine	0.45	2.35	0.31	1.80
DL phenylalanine	0.12	0.90	0.09	0.45
L proline	0.00	0.00	0.00	0.00
DL threonine	0.00	0.00	0.00	0.00
L tyrosine	0.02	0.00	0.00	0.70
DL valine	0.00	0.00	0.00	0.00
DL tryptophane	0.00	0.00	0.00	0.00
L cysitine	0.12	1.02	0.19	0.25
DL serine	0.45	2.04	0.00	0.00
glycine	0.00	0.00	0.00	0.00
L leucine	0.00	0.00	0.00	0.00

Assays were made after three days' culture.

vorin was not needed. This independency on leucovorin is a distinct character of *Ped. acidilactici* differentiating the species from other pediococci.

These nutritional specificities of this strain closely resemble the results obtained from other strains of the same species like so-called *Leuconostoc mesenteroides* P-60[14~16]. Nakagawa and Kitahara's strain reported that *Ped. acidilactici* required riboflavine, nicotinic acid, pantothenic acid, biotin but does not require pyridoxine. These results almost agree with these of the author's, with the exception of the unnecessity of pyridoxine. This discrepancy might be due to their long culture period or to the basal medium containing casamino acids as the amino acid source.

Ped. acidilactici Kitahara's strain requires all the amino acids except methionine. For strain P-60, the requirements for all the amino acids except alanine were reported by Dunn[17] and Steele[18], and this character made this strain useful for bioassay of amino acids among other many species of lactic acid bacteria.

This character requiring almost all amino acids for growth is the specificity of salt-sensitive pediococci, both *Ped. acidilactici* and *Ped. pentosaceus,* which differentiate them from other lactic acid bacteria and even salt-tolerant pediococci. This phenomenon suggests that these two species do not have transaminase, or are not activated even in keeping the enzyme in the cell. The existence of activated transaminase in cells of salt-tolerant pediococci is an interesting problem deserving future research.

Acknowledgements The author wishes to express his sincere thanks to Prof. T. Asai and Prof. K. Kitahara, University of Tokyo for their kind guidance and generous supply of precious strains. Thanks are also due to Director M. Mogi and members of this Institute for their kind advice. He is also especially grateful to Mr. T. Kuramochi for his earnest and able assistance.

Studies on the Mechanism of Mold Protease Production*

Part II. Inactive Form Protease in Cell free Extracts of *Aspergillus sojae* KS and its Activating Factors**

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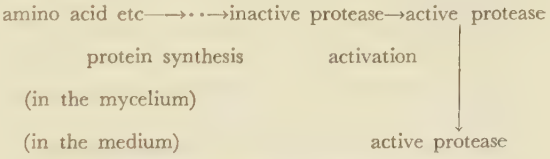
Received November 26, 1960

Inactive protease in the cell free extracts obtained from growing cells of *Aspergillus sojae* KS was collected in a supernatant of ultracentrifugation at 14×10^4 g, and in fractions obtained by acetone of 35~50 per cent and by ammonium sulfate of 0.5~0.6 saturation.

The inactive protease has the same resistance against pH or heat treatments as active protease has. The activation of the inactive protease was maximal between pH 5~6, and was accelerated by several kinds of protease, and was not affected by thioglycollate and KCN.

In the previous report[1], it was recognized that the protease activty in a cell free extract, which was otbained by extracting mashed mycelia of *Aspergillus sojae* KS with distilled water, increased during its incubation. Different from other proteases formed by this mold, the increased protease in question turned out to be an alkaline protease, as is known from the fact that its increase was inhibited by potato-inhibitor. It was found that the cell free extract which increased protease activity to a high degree was the extracted from a mold in early period of log. phase growth. To be more accurate, it is especially in the early phase of spore-producing period of the mold (which is also a phase when the mold is beginning to secrete alkaline protease into medium) that the most active cell free extract can be obtained.

It has also been proved that the increase of protease activity mentioned above is not due to new protein being synthesized, because the increase was not affected by inhibitors of protein snythesis. It is observed further that the increase of protease was found to be subject to the influence of temperatur. From these results it was suggested in the previous paper[1] that zymogen (inactive protease) is formed in the course of formation of alkaline protease by the mold, and that this material become activated during incubation, as is shown in the following scheme:



The Scheme of Alkaline Protease Formation by *Aspergillus sojae* KS

The following report deals more fully with the existence of inactive protease in the cell free extract of *Aspergillus sojae* KS, and with activating effects on the inactive protease.

* Reprinted by permission of Agr. Chem. Soc. Japan from Agr. Biol. Chem. Vol. 25, No. 6.

** Presented at the Meeting of the Agricultural Chemical Society of Japan, Tokyo, July 19, 1958.

1) K. Yamamoto, This Journal, **23**, 110 (1959).

MATERIALS AND METHOD

1. Strain Used. *Aspergillus sojae* KS was chosen as was mentioned in the previous report[1].

2. Preparation of a Cell Free Extract. Growing cells, obtained from surface culture on a medium as is shown in Table I, were washed well and pressed with layers of cloth. Then it was treated with 20 volumes of clod acetone at −10°C in order to remove water, and dried with P_2O_5 in a vacuum. Resultant dried mycelia were extracted with 10 to 20 volumes of distilled water.

Table I. Composition of Medium

Component	g/l
Glucose	50
Peptone	30
KH_2PO_4	5.0
$MgSO_4$	0.1
$ZnCl_2$	0.02
$MnSO_4$	0.02
$FeCl_3$	0.02
NaOH	7.0
Initial pH	7.0

3. Incubation of the Cell Free Extract. After the cell free extract mentioned above was adjusted to various pH's, or after various inhibitors or proteases were added to it, the cell free was incubated at 30°C. Then the protease activity in the cell free extract was determined at intervals by the usual method[2].

EXPERIMENTAL RESULTS

1. Fractionation of Cell Free Extracts by Ultracentrifugation.

Immediately after a cell free extract was prepared, the cell free extract was fractionated by ultracentrifugation of Spincto type. As is shown in Table II, that portion of the extract which was capable of increasing protease activity during the incubation as well as active protease existent from the beginning in the cell free extract was nearly completely collected in the supernatant of ultracentrifugation at 14×10^4 g On the other hand sediments obtained through the same process did not increase its protease activity, not even when some portion of the supernatant, after protease activity in it had stopped to increase, was added to the sediments and incubated at various pH's.

Table II. Effect of Fractionation by Ultracentrifugation on the Increase of Protease Activity in the Cell Free Extract of *Aspeagillus sojae* KS.

Condition of centrifugation (0°C, 10 mins.)	Incubation time after centrifugation		
	0	4 hrs.	6 hrs.
control	0.032	0.123	0.195
3×10^4g, supernatant	0.032	0.132	0.206
6×10^4g, //	0.047	0.125	0.185
14×10^4g, //	0.058	0.145	0.201
// sediment	0.002	0.001	0.006

The cell free extract used was prepared as follows: 5 g of acetone-dried mycelia obtained from growing cells of *Aspergillus sojae* KS at 30°C for 48 hours was extracted with 75 ml of distilled water. After centrifugation of the cell free extract each supernatant and the solution of 15 ml of distilled water, by which the sediment of centrifugation at 14×10^4g was suspended, were incubated at 30°C. The preparation of cell free extract is common to Fig. 2 and 3, and to Table III, IV, V, VI, VIII and IX.

If that part of cell free extract which caused activation was related to mycelial particulates, it would have been collected in the sediments of ultracentrifugation at 14×10^4 g. Therefore the increase of protease activity in the cell free etxract was proved not due to active alkaline protease which, once adsorbed by mycelial particulates, has been eluted again during the incubation of the cell free extract.

The previous report[1] showed that the increase of protease activtiy in the cell free extract was not caused by synthesis of new protein.

Therefore the cause for the increase of protease activity must be found in the existence of inactive form protease in cell free extract of *Aspergillus sojae* KS. And this inactive protease with its activating factors is collected in the supernatant obtained through ultracentrifugation at 14×10^4 g.

2. Fractionations of Inactive Protease in Cell Free Extracts by Acetone and by Ammonium Sulfate.

In order to ascertain existence of inactive form protease in it, the cell free extract was fractionated by ammonium sulfate and by acetone.

By this procedure the active protease and the part containing inactive protease were observed to precipitate in the fraction obtained through from 0.5 to 0.6 saturation of ammonium sulfate as well as in the fraction obtained through from 35 to 50 per cent of acetone (Table III).

Incidentally the factor to activate inactive protease in cell free extract was assumed to precipitate in the

2) K. Yamamoto, This Journal, **21**, 313 (1957).

Table III. Fractionations of Active and Inactive Protease in Cell Free Extract by Acetone and by Ammonium Sulfate

Fraction	Active protease (%)	Inactive protease (%)
control (original)	100	100
0~35%, acetone	16	14
35~50%, //	75	55
50~70%, //	10	5
70~90%, //	2	0
0~0.5 sat., ammonium sulfate	25	5
0.5~0.6 sat., //	65	70
0.6~0.7 sat., //	5	2
0.7~0.9 sat., //	2	0

Inactive form protease in cell free extract and in each fraction was calculated as increased protease activity during incubation of the cell free extract and the solutions of each fractions at pH 7.0 for 6 hours.

same fractions, because spontaneous increase of protease activity in solutions of these fractions was observed during their incubations.

3. Paper Electrophoresis of Cell Free Extracts.

To separate inactive protease, active protease, and activating factors from each other, the cell free extract was subjected to paper electrophoresis. Paper electrophoretic apparatus of the Tōyō Roshi type and the Tōyō Roshi No. 52 electrophoresis paper strip was employed. The electrophoresis was carried out in 0.025 M phosphate buffer, at pH 8, and the other conditions were as follows: a 2 cm strip was run with constant field strength of 250 volts at 10°C for 8 hours; the cell free extract used was a solution of a precipitate

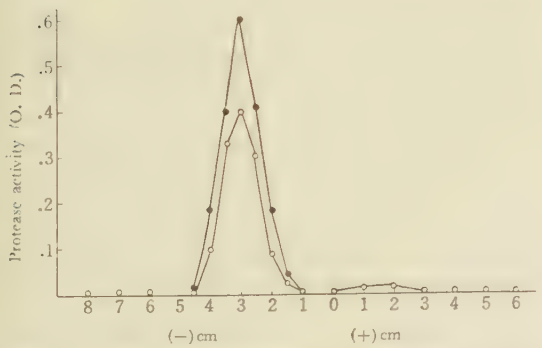


Fig. 1. Paper Electrophoresis of a Cell Free Extract.

For experimental conditions see text.

○——○ initial protease activity.

●——● increased protease activity during each fraction's incubation at pH 7.0, for 5 hours., at 30°C.

obtained through fractionating from 35 to 50 per cent of acetone; after electrophoresis, the paper strip was divided into pieces each 2 cm long; eluates obtained from each piece were incubated at 30°C for 5 hours; protease activities of extracts before and after the incubation were measured. As is shown in Fig. 1, it was found that active alkaline protease and inactive protease were found to have migrated to the same position on cathode side of the paper. And the inactive protease and its activating factors could not be separated from each other by the electrophoretic process.

4. Effect of Heat Treatment on Alkaline Protease and on Inactive Protease in Cell Free Extracts.

Ten ml of the cell free extract were incubated in a constant temperature water bath, which was kept at 40°C, 50°C, and 60°C in turn, but always at pH 7.0 for 15 minutes. Then activities of both active and inactive protease in each case were determined. The results expressed in percentage are shown in Table IV taking the activity remained in the case of keeping water bath at 30°C as 100 per cent. In the case of incubation at 60°C both active alkaline protease and the inactive protease in the cell free extract were one hundred per cent inactivated. The original cell free extract which had stopped increasing protease activity was added to the cell free extract treated at 60°C, but the increase of protease activity did not occur.

Table IV. Effectc of Heat, Alnaline, and Acid Treatment on the Active and Inactive Protease in Cell Free Extract

Condition of treatment	Active protease remaining (%)	Inactive protease remaining (%)
control	100	100
40°C, 15 mins	100	100
50°C, //	60	60
60°C, //	0	0
pH 10, 30°C, 10 mins.	95	95
pH 10.5, //	90	90
pH 11, //	80	85
pH 11.5, //	20	25
pH 12, //	0	0
pH 4.5, //	65	74
pH 4.0, //	38	50
pH 3.5, //	25	37
pH 3.0, //	0	0

Inactive protease in cell free extrat was calculated by the same method sa is shown in Table III.

5. The Effect of Acid and Alkali Treatment on Active Protease and Inactive Protease in Cell Free Extracts.

Ten ml of the cell free extract was incubated at 30°C for 10 minutes at various pH's of both acid and alkali. Then activities of both active and inactive protease in each case was determined. The results are shown in Table IV with remaining activity in the case of p H7 as a basis (100 per cent). A hundred per cent inactivation of both alkaline protease and the niactive protease was observed in the case of incubation at pH 12 as well as at pH 3.0. After this treatment the original cell free extract, which had stopped increasing protease activty, was added. But the process proved to have no effect on increasing protease activity in the treated cell free extracts again.

6. Effect of Incubation pH upon the Increase of Protease Activity in the Cell Free Extracts.

After extraction, the cell free extract was adjusted separately to various pH's without delay and incubated at 30°C. Then protease activity in each cell free extract was determined. As is shown in Fig. 2 increase of protease activity was observed in a wide range of pH.

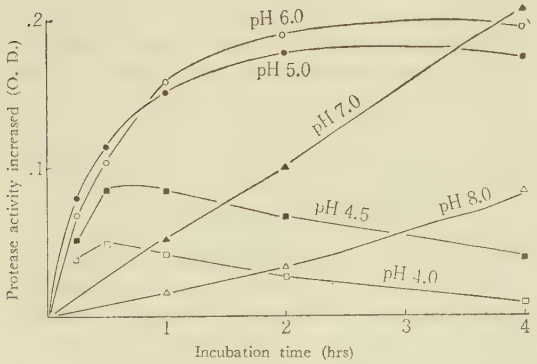


Fig. 2. Effect of Incubation pH on the Increase of Protease Activity in Cell Free Extract.

The increase of protease activity was observed to have occurred most rapidly between pH 5 and 6, and most slowly at alkaline pH's. The increased protease was proved to be alkaline protease in every case by means of potato-inhibitor (Table V). In order to confirm the results obtained, measurement of protease activity was made at pH 3.5. In this case the increase of protease activity was not observed (Table V).

Table V. Inhibition of Increased Protease in Cell Free Extract by Potato-inhibitor and Activity of Increased Protease Measured by Digestion of Casein of PH 3.5

Incubation pH of cell free extract	Potato-Inhibitor (after incubation	Enzymation pH	Incubation time (hrs.) 0	3	6
5	none	7	0.057	0.201	0.198
	added	〃	0.02	0.015	0.010
6	none	〃	0.055	0.223	0.205
	added	〃	0.035	0.040	0.030
7	none	〃	0.058	0.125	0.243
	added	〃	0.040	0.040	0.039
5	none	3.5	0.020	0.024	0.021
6	〃	〃	0.020	0.020	0.015
7	〃	〃	0.020	0.015	0.010

After incubation of cell free extracts at various pHs, each cell free extract was pre-incubated at 30°C for 10 minutes, with and without potato-inhibitor.

This fact shows clearly that the increase of protease activity in the cell free extract is not due to increase of acid protease produced by the mold used.

7. Effect of Neutral Protease Produced by *Aspergillus oryzae* on the Activation of Inactive Protease in the Cell Free Extract.

Judging from the experimental results that the most rapid increase of protease activity was observed when incubated at pH 5~6, it is assumed that neutral protease, which has been reported to be produced by *Aspergillus oryzae*[3~7] and *Aspergillus sojae*[2], has some influence on activation of the supposed inactive protease. As the next step, therefore, effect of neutral protease on the increase of protease activity in the cell free extract was investigated.

As Fig. 3 shows, neutral protease was observed to be able to promote increase of protease activity in the cell free extract, but when a cell free extract was incubated with EDTA (final concentration of 10^{-2} M) the same amount of increase of protease activity as in the case of control incubation without EDTA was observed. The reason for this phenomenon will be (a) that the neutral protease is not an essential activator of inactive protease and does accelerate the spontaneous activation, or (b) that another protease

3) T. Yasui, Lectured at the Meeting of Agr. Chem. Soc. Japan, (1955), (1956).
4) M. Suzuki, Y. Nunokawa and Y. Ito, *J. Agr. Chem. Soc. Japan*, **33**, 857 (1959).
5) K. Katsushima, *ibid*, **33**, 116, 120 (1959).
6) Y. Miura, *ibid.*, **32**, 486 (1958).
7) H. Specht, *Naturwissenschaften Heft.*, **2**, 37 (1957).

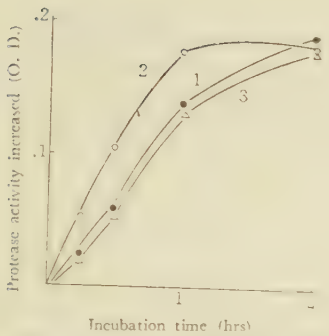


FIG. 3. Effect of Neutral Protease and EDTA on the Increase of Protease Activity in Cell Free Extract.

The cell free extract were incubated at pH 6.0, at 30 C. Curve 1, Control; Curve 2, with neutral protease (0.05 mg/cc) prepared by YASUI; Curve 3, with 2×10^{-2}M EDTA.

hitherto unknown and existent in partially purified neutral protease is a factor activating inactive protease.

8. Effect of Sodium Lauryl Sulfonate (S.L.S.) on the Activation of Inactive Protease in the Cell Free Extract.

Sodium lauryl sulfonate, known as a specific inhibitor[8,9] of acid protease produced by the mold, was added to the cell free extract, and the increase of protease activity was determined after the cell free extract was incubated at pH 6 or 7. As a result, the same amount of increase of protease activity was obtained both in the cell free extract with S.L.S. and in the cell free extract without S.L.S. (Table VI). When

TABLE VI. EFFECT OF SODIUM LAURYL SULFONATE ON THE INCREASE OF PROTEASE ACTIVITY IN CELL FREE EXTRACT

S.L.S. (final conc. of 10^{-2}M)	Pre-incubation pH	Incubation pH	Relative activity of increased protease (%)
none	7.0	7.0	100
abbed	//	//	100
none	6.0	6.0	100
added	//	//	90
//	4.5	//	74
//	4.0	//	50
//	3.5	//	37
//	3.0	//	0

The cell free extract with 10^{-2}M S.L.S. was pre-incubated at separate pH for ten minutes and incubated at pH 6.0 or 7.0 at 30 °C for 5 hours.

Figures show as percentage with maximum protease activity increased in cell free extract without S.L.S. as 100 per cent.

8) F. Yoshida and M. Nagasawa, This Journal, **20**, 257 (1956).
9) N. Iguchi and K. Yamamoto, *J. Agr. Chem. Soc. Japan*, **29**, 387 (1955).

TABLE VII. EFFECT OF SODIUM LAURYL SULFONATE ON THE THREE KINDS OF PROTEINASE PRODUCED BY KOJI MOLDS

Kind of protease	S.L.S.	Pre-incubation pH	Relative activity of protease (%)
Asp. sojae alkaline protease (by Mizunuma	none	7.0	100
	added	7.0	95
	//	5.5	80
	//	5.0	0
	//	3.5	0
Asp. oryzae neutral protease (by Yasui)	none	7.0	100
	added	7.0	95
	//	5.0	0
	//	3.5	0
Asp. Saitoi acid proteaes (by Yoshida)	none	5.0	100
	added	5.7	44[62]
	//	4.5	0[23]
	//	3.5	0[15]
	//	2.5	0[0]

[ ]: show the relative protease activity in the case of crude enzyme preparation.
( ): kindly donated by them.

the incubation of the cell free extract with S.L.S. at pH 3~5, optimal pH range for acid protease, was undertaken, active alkaline protease was inactivated (Table VII). Accordingly it was difficult to determinde the effect of S.L.S. on the activation of inactive protease in the cell free extract incubated at pH 3~5. Then, after being treated with S.L.S. of final concentration of 10^{-2}M at pH 3~5, the treated cell free extract was incubated at pH 6.0. As is shown in Table VI, the cell free extract treated with S.L.S. at pH 3.5 was still able to increase protease activity, while the acid protease was inactivated through the treatment with S.L.S. at the same pH. These results show that the acid protease is not the essential activator of the inactive protease in the cell free extract.

9. Effect of Various Kinds of Protease on the Activation of Inactive Protease in the Cell Free Extract.

After various kinds of protease were added to the cell free extract, protease activity in the cell free extract was measured at intervals.

As is shown in Table VIII, *Aspergillus Saitoi* acid protease, Taka diastase, papain and pepsin had very little effect on the increase of protease in the cell free extract. Carboxypeptidase, trypsin, Nagase (bacterial protease), and extract from rice koji of *Aspergillus oryzae* MP-39, howevr, had accelerating effect on the activation of inactive protease in the cell free extract. But it is not clear whether these protease are essential for the activation of inactive protease or have only accelerating effect on the spontaneous activation.

Table VIII. Eeefcts of Various Kinds of Protease on the Increase of Protease Activity in Cell Free Extract

Kind of protease	Incubation pH	Incubation time (hrs.) 1(0.5)	2(1)	4(2)
Control	7.0	0.038	0.073	0.120
Trypsin	//	0.065	0.110	0.130
Nagase (bacterial protease)	//	0.040	0.100	0.125
Asp. sojae alkaline protease	//	0.043	0.071	0.115
Carboxypeptidase	//	0.059	0.132	0.125
Chymotrypsin	//	0.063	0.108	0.131
Control	5.5	0.040	0.065	0.081
Papain	//	0.052	0.060	0.082
Pepsin	//	0.038	0.055	0.093
Taka-diastase	//	0.030	0.060	0.080
Asp. Saitoi acid protease	//	0.030	0.060	0.080
Rice koji extract*	//	0.068	0.099	0.105

( ) : show a incubation time in the case of incubation of pH 5.0.

*: obtained fiom rice koji of *Aspergillus oryzae* MP-39.

This is because the cell free extract used had not been sufficiently purified beforehand and might have contained an activating factor in itself as well as inactive protease.

10. Effect of Several Kinds of Substance on the Activation of Inactive Protease in the Cell Free Extract.

Several kinds of substance as shown in Table IX were added to the cell extract, and then protease activity increased in each case was measured after its incubation at pH 7, at 30°C for 5 hours. However,

Table IX. Effects of Various Kinds of Inhibitor on the Increase of Protease Activity in Cell Free Extract

Substance added	Final concentration	Relative activity of increased protease (%)
KCN	10^{-2}M	113
Na_2S	//	101
$NaNO_2$	//	114
Arsenate	//	106
Monoiodo-acetate	//	106
NaF	//	100
Trypsin-inhibitor	0.001%	104
Thioglycolate	0.01 %	100
// (in vacuume)	//	115

these substances had on effect on the activation of inactive protease in the cell free extract, as is known from the fact that the same amount of increase of protease activity was observed in the cell free extract with those substances added as in the case of a controlled cell free extract without any of those substances. The fact that KCN, Na_2S, and thioglycollate had no effect on the increase of protease activity in the cell free extract shows that the activation of inactive protease does not involve the change of SH group.

11. A Protease Remaining in Two Kinds of Koji Extract after Treatment with S.L.S. at pH 4.0.

As mentioned above, it is supposed that there may exist a new protease having kinase effect on inactive protease at pH 5∼6 optimum.

Accordingly, after enzyme solutions obtained from a rice koji culture of *Aspergillus oryzae* MP39 and from a wheat bran culture of *Aspergillus sojae* KS were treated with S.L.S. at pH 4.0, the pH activity curves of the protease in these enzyme solutions were determined. The experimental results show that the pro-

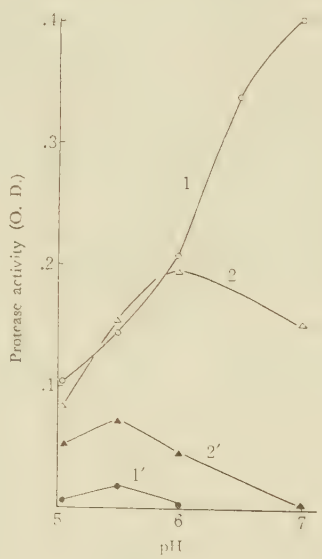


Fig. 4. pH Activity Curves of Two Kinds of Koji Extract with and without S.L.S.

Curve 1, extract from wheat bran koji of *Asp. sojae* KS, no treatment.

Curve 1′, extract from wheat bran koji of *Asp. sojae* KS, treatment with S.L.S. at pH 4.0.

Curve 2, extract from rice koji of *Asp. oryzae* Mp-39, no treatment.

Curve 2′, extract from rice koji of *Asp. orpzae* Mp-39, treatment with S.L.S. at pH 4.0.

tease having its optimal activty at pH 5~6 is unaffected by inactivating effect of S.L.S., while acid, neutral, and alkaline proteases were inactivated by such treatment (Fig. 4). When these enzyme solutions were incubated with potato-inhibitor (inhibitor of alkaline protease) and EDTA (inhibitor of neutral protease), the protease activty proved to be unaffected between pH 5 and 6.

DISCUSSION

Schneider and Hogeboom[12], and Berthet et al.[13] have reported that the activity of enzymes in intact liver particulate is low and that maximal activity in such preparation is attained when the particulate matter is disrupted or undergoes a disintegration due to unfavorable environment.

Thus, the increase of protease activity in the cell free extract could be due to release of active alkaline protease from particulate matter in the cell free extract undergoing a gradual disruption.

Through ultracentrifugation of the cell free extract at 14×10^4 g, the fraction capable of increasing protease activity was collected in the supernatant. Accordingly, the increase of protease activity in the cell free extract was not due to the fact that active alkaline protease already formed in mycelia was adsorbed into the mycelial particulate, and that the protease adsorbed was eluted again into the cell free extract during its incubation. And there may exist in the supernatant both inactive protease and its activating factors, because the protease activity in the supernatant increased spontaneously during incubation. The following experimental results were established; 1) the fraction capable of increasing protease activity was collected in the precipitant obtained by 0.5~0.6 saturation of ammonium sulfate and also in the precipitant obtained by 35~50 per cent of acetone; 2) the fraction mentioned above migrated to the same position on a paper of eletcrophoresis test as active alkaline protease; 3) it showed almost the same degree of resistance against three kinds of treatment (acid, alkaline, and heat) as active alkaline protease. Thus it was confirmed that there exist in the cell free extract inactive form portease similar in nature to active alkaline protease and that this inactive protease becomes activated during incubation of the cell free extract, spontaneously or by activating factors.

What sort of activation occurs in the cell free extract? It is generally agreed upon that there are three types of activation of protease zymogens. These are 1) zymogen is autocatalytically activated by the protease formed by the same zymogen[14~16], 2) zymogen is activated by other protease[17~21] than one formed by the same zymogen, and 3) zymogen is activated by the change of hydrogen ion concentration[22,23] or by oxidation-reduction[24] process, and not enzymatically. The velocity of activation of inactive protease became maximal between pH 5 and 6, while the active alkaline protease had its maximal activity between pH 7 and 10[9].

Moreover addition of pure alkaline protease to the cell free extract had no effect on the increase of protease activity in the cell

* This fact was observed also with several kinds of mold by Matsushima[5], and he named "semi acid protease" for such protease, but it is not be sure the existence of "semi acid protease."[10, 11].

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free extract as was shown in the previous report[1]. Accordingly, the type of activation in question is supposed not to be autocatalytic as in the case of trypsinogen[14] being activated by trypsin itself. Next, the activation of inactive protease is assumed not to be due to dessociation of active protease inhibitor complex only by change of pH as in the case of activation of prorennin[23] and pepsinogen[22], because it was observed that the protease activity in the cell free extract increased in such a wide range of pH from acid to alkaline. It is probable (a) that neutral protease is not essential for the activation of inactive protease because addition of EDTA to the cell free extract did not have any effect on the increase of protease activity in the cell free extract and (b) that acid protease is not essential for the activation in question because the cell free extract could increase its protease activity after being treated with S.L.S. at pH 4.0. That treatment at pH 4.0 must have inactivated acid protease. Judging from the results that the addition of thioglycollate and KCN to the cell free extract had no effect on the increase of protease activtiy in the cell free extract, an oxidation-reduction process can not be related to the activation.

Of several kinds of protease added to the cell free extract, Nagase, carboxypeptidase, trypsin, chymotrypsin, rice koji extract of *Aspergillus oryzae* MP-39 and partially purified neutral protease had an accelerating effect on the activation. However, such an essential effect as is observed in the case of kinase activating trypsinogen[17~19], chymotrypsinogen[20], or procarboxypeptidase[21] was not observed in this case, because some extent of activating factor of inactive protease is supposed to exist in the cell free extract from the beginning or the inactive protease in question might have a nature of being activated spontaneously and therefore promoting effect of proteases added was not so marked as the cases above.

Accordingly, in order to identify the protease having an essential effect for the activation of inactive protease, it is necessary to use more purified one having less activating effect in itself.

Acknowledgement The author wishes to express his sincerest thanks to Emeritus Prof. K. Sakaguchi, Prof. K. Arima and Prof. B. Maruo of University of Tōkyō for their kind guidance.

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Studies on the Mechanism of Mold Protease Production*

Part III. Activating Factors of Inactive Protease in Cell-Free Extracts of *Aspergillus sojae* KS**

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The activating factors of the inactive protease in cell-free extracts obtained from growing mycelia of *Aspergillus sojae* KS were studied. It was found that the several kinds of metals were involved in activation, and the role of these metals on the activation was investigated. The velocity of the activation was maximal around pH 10 as well as around pH 5. It was proved that a kinase (enzyme) capable of activating the inactive protease in alkaline solution does exist in the cell-free extract.

In the previous report[1], it was shown that an inactive protease was formed in the course of formation of alkaline protease by *Aspergillus sojae* KS, and the several kinds of factors in the cell free extracts acting on the inactive protease were investigated. It was found that the inactive protease was not autocatalytically activated either by the change of hydrogen ion concentration or by the oxidation-reduction process.

From the experimental results that the velocity of activation was maximal at pH 5~6 and that the activation was accelerated by some kinds of protease, it was supposed that there existed a kinase capable of activating the inactive protease. It was not possible, however, to isolate such a kinase from the inactive protease preparation and possibility remained that the inactive protease might have been activated spontaneously and not enzymatically.

Recently, Gjessing et al[2] have reported that an esterase exists as a precursor in hog pancreas and that the spontaneous activation appears to be enzymatically controlled. It has been recognized that the activation is markedly dependent upon temperature, ionic strength, and pH with an optimum between 5 and 6, and that the addition of trypsin accelerates the activation.

Tôyama et al[3,4] repeated the present author's previous experiments and confirmed the presence of zymogen precursor in the course of protease production by *Aspergillus oryzae* W-1 and suggested that the activation of proenzyme was not due to the presence of a kinase, but was a spontaneous phenomenon at a pH below 7.5.

In the present communication the activation of inactive protease in cell free extracts of the *Aspergillus sojae* KS was further investigated.

As results, it was recognized that several

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** Presented at a meeting of the Agricultural Chemical Society of Japan, Tokyo, September 24, 1960.

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kinds of metals are involved in the activation of inactive protease and that the optimum pH of activation lies between 9 and 10 as well as between 5 and 6, and the inactive protease is apparently activated at an alkaline pH by some enzymes.

MATERIALS AND METHOD

1. Strain Used. *Aspergillus sojae* KS was used as is mentioned in the series of papers "Studies on Koji".

2. Preparation of Cell Free Extracts. Cell-free extracts were prepared from growing mycelia obtained from cultures on two kinds of media as are shown in Table I, according to the method described in the previous paper.

TABLE I. COMPOSITION OF GROWTH MEDIA

	M-Medium(g/l)	K-Medium(g/l)
Glucose	50	10
Peptone	30	7.5
KH_2PO_4	5	5
$MgSO_4$	0.1	0
KCl	—	5
$ZuCl_2MnCl_2FeCl_2FeCl_3$	0.02	—
NaOH	7	—
Initial pH	7.0	5.6

3. Determination of the Activity of Active Protease and Inactive Protease in Cell Free Extracts. Immediately after extraction, the protease activity in a cell free extract was determined by the usual method.

After the cell free extract was incubated at a constant pH at 30°C for several hours, the protease activity in the cell free extract was again measured.

The increase of protease activity during the incubation of cell free extract was calculated as inactive protease activity.

EXPERIMENTAL RESULTS

1. Effect of EDTA on the Activation of Inactive Protease in Cell Free Extracts.

As is shown in Fig. 1, when a cell free extract was incubated with EDTA (10^{-2} M) at 30°C the increase in protease activity of the cell free extract was the same as that of the cell free extract without EDTA. When the cell free extract was dialyzed against EDTA at 0°C, however, the activation of inactive protease in the cell free extract was markedly suppressed. EDTA

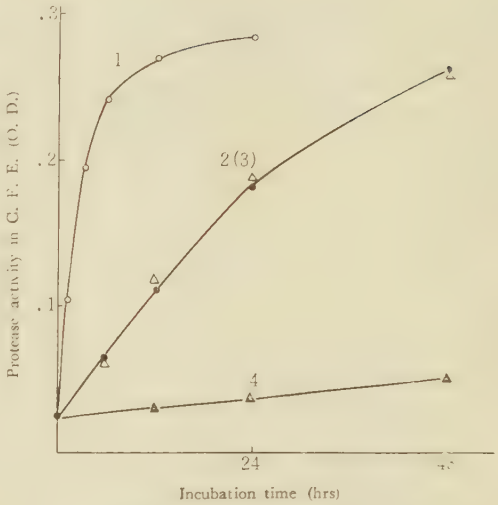

FIG. 1. Effect of Dialysis on the Increase in Protease Activity of Cell Free Extract.

The cell free extract was obtained from mycelia of *Aspergillus sojae* KS at 30°C for 36 hours on K-medium. After the cell free extract was incubated or dialyzed at pH 7.0, the protease activity in cell free extract was measured at intervals. Curve 1, incubation at 30°C; curve 2, incubation at 0°C; curve 3, dialysis against distilled water at 0°C; curve 4, dialysis against 2×10^{-2} M EDTA at 0°C.

TABLE II. EFFECT OF DIALYSIS AGAINST VARIOUS SUBSTANCES ON THE INCREASE OF PROTEASE IN CELL FREE EXTRACT DURING THEIR INCUBATION

Dialysis against (pH 7.0, 0°C)	Incubation time (hrs.)			
	0	14	24	36
Distilled water	0.056	0.102	0.183	0.234
8-Hydroxyquinoline	0.061	0.059	0.125	0.149
EDTA	0.019	0.019	0.021	0.025
l-Ascorbic acid	0.025	0.031	0.032	0.042

was the most effective of all the metal chelating compounds tested which showed the suppressing effect on the activation of inactive protease (Table II).

When the cell free extract was dialyzed against distilled water at 0°C, the increase curve of protease activity of the cell free extract was similar to that of the cell free extract incubated at 0°C without dialysis.

2. Effect of Several Kinds of Metallic Ions on the Activation of Inactive Protease in the Cell Free Extract.

In order to investigate the inhibition of inactive

protease activation by EDTA, various kinds of metallic ions were added to the cell free extract, which had been dialyzed against EDTA at 0°C for 24 hours. The cell free extract was then incubated at 30°C and the increase of protease activity was followed. As is shown in Table III, many kinds of metallic ions had ability to recover the suppressing effect on the activation of the inactive protease by EDTA. Especially, such metallic ions as Cu^{++}, Ni^{++}, Pb^{++} and Zn^{++} showed the highest recovery effect. These metallic ions had also an accelerating effect on the activation of inactive protease in the cell free extract which had not been dialyzed against EDTA (Table IV).

TABLE III. RECOVERY OF ACTIVATION ABILITY OF PROTEASE IN CELL FREE EXTRACT BY VARIOUS METALLIC IONS AFTER DIALYSIS AGAINST EDTA

Metal added	Incubation time after dialysis against EDTA (hrs.)		
	0	4	8
Control	14	21	33
Ca^{++}	15	34	59
Co^{++}	17	53	73
Cu^{++}	43	86	100
Mg^{++}	11	25	61
Mn^{++}	19	32	63
Pb^{++}	15	39	65
Zn^{++}	18	34	61
Fe^{++}	18	26	56
Ni^{++}	31	63	78
Sr^{++}	15	19	26
Li^{+}	12	25	47
Ba^{++}	20	39	63

The cell free extract was incubated with various metals at pH 7.0 at 30°C. Before the additions of metallic ions the cell free extract was dialyzed against 2×10^{-2} M EDTA at pH 7.0, at 0°C, for 48 hours.

Metals were added in the form of chloride salts. Figures shown represent the percentages of the maximum activity obtainable under these conditions.

TABLE IV. EFFECT OF VARIOUS METALIC IONS ON THE INCREASE OF PROTEASE ACTIVITY IN CELL FREE EXTRACT

Metal added	Incubation time (hrs.)		
	0	3	7
Control	13	57	91
Ca^{++}	14	58	86
Cu^{++}	51	84	100
Mg^{++}	23	54	98
Co^{++}	20	43	78
Mn^{++}	23	56	85
Pb^{++}	15	48	65
Zn^{++}	25	80	100
Ba^{++}	13	46	84
Fe^{++}	10	27	76
Ni^{++}	30	64	100
Li^{+}	19	62	85
Cu^{+}	74	100	100
Fe^{+++}	20	48	65

The cell free extract was incubated with various metals at pH 7.0, at 30°C. Other conditions were as described in Table III.

3. Effect of EDTA and Metallic Ions on Active Alkaline Protease.

After the activation of inactive protease the cell free extract was treated with EDTA at 30, 40, and 50°C,

TABLE V. EFFECT OF EDTA ON THE HEAT TREATMENT OF ACTIVE PROTEASE

Temperature of treatment	EDTA (final conc. of 10^{-2} M)	Time of treatment (mins.)			
		0	15	30	60
30°C	absent	100	—	—	100
	present	90	—	—	90
40°C	absent	100	—	100	80
	present	90	—	90	90
50°C	absent	100	30	15	
	present	90	40	20	

Figures are shown as percentage of protease activity in cell free extract with original protease activity in the cell free extract taken as 100 per cent.

TABLE VI. EFFECT OF VARIOUS METALS ON THE PROTEASE ACTIVITY IN CELL FREE EXTRACT AFTER COMPLETION OF ACTIVATION

Metal added	Incubation time (hrs.)		Final pH
	0	3	
Control	100	100	7.0
Ca^{++}	88	90	7.0
Co^{++}	90	95	7.0
Cu^{++}	100	110	7.0
Mn^{++}	120	130	6.8
Pb^{++}	70	85	6.0
Zn^{++}	80	90	6.6
Fe^{++}	80	100	6.9
Ni^{++}	70	75	6.4
Li^{+}	100	100	6.8
Ba^{++}	80	100	6.5

Various metals were added to cell free extract after the increase of protease activity had been finished, and the cell free extracts were incubated at 30°C, at pH 7.0. Figures are shown as percentage of protease activity in cell free extract with maximal activity in cell free extract without metals as 100 per cent.

for 30 and 60 minutes. As is shown in Table V, the protease in cell free extract in the presence of EDTA had the same degree of resistance against heat treatment as that in the absence of EDTA. Purified alkaline protease prepared by Mizunuma's[5] method showed also similar resistance against such treatment, and was not inactivated by dialysis against EDTA for a week at 0°C.

Next, metallic ions having the accelerating effect on the inactive protease activation were added to the cell free extract in which inactive protease had been fully activated. As is shown in Table VI the metallic ions in question had no effect on the active protease in the cell free extract after activation of inactive protease had been finished.

4. Effect of pH on the Activation of Inactive Protease in the Cell Free Extract.

As was mentioned in previous report[1], the activation of inactive protease was most rapid between pH 5 and 6. But, it was often recognized during chromatography of a cell free extract by DEAE cellulose that the inactive protease in the cell free extract was rapidly activated in an alkaline solution above pH 9. Therefore, in order to investigate again the effect of pH on the activation of inactive protease, a fresh preparation of the cell free extract obtained from growing cells of culture on M-medium was incubated at various pH's at 30°C for 15 minutes.

FIG. 2. Effect of pH of Incubation Medium on the Protease Activity in Cell Free Extract.

The cell free extract was incubated at various pH's at 30°C for fifteen minutes, and protease activity in the extract was determined for casien at pH 7.0 as described elsewhere.

As is shown in Fig. 2, the optimum pH of activation was found to be near pH 10 as well as near pH 5.

Protease activity increased during incubation of the cell free extract either at pH 5 or pH 10 was proved to be due to an alkaline protease by the test of potato-inhibitor.

5. Effect of Cultural Conditions on the Activation of Inactive Protease in the Cell Free Extract.

In order to confirm Tōyama's experimental results[4] that the activation of inactive protease did not occur in a solution at a pH above 7.5, two kinds of cell free extract were prepared from mold mycelia grown on K and M media and the activation of inactive protease in two kinds of cell free extract was compared. As is shown in Table VII the cell free extract (early K.C.F.E.) obtained from mycelia at an early period of culture on K-medium was found to be incapable of increasing protease activity during its incubation at pH 8.5, and the cell free extract (later K.C.F.E.) prepared from mycelia at a later period of culture on K-medium had apparently the ability to increase protease activity during its incubation at pH 8.5. Of course, the cell free extracts (M.C.F.E.) prepared from mycelia at all periods of culture on M-medium had the ability to increase protease activity during their incubation at pH 8.5. In order to investigate whether, under any condition, the inactive protease in the early K.C.F.E. cannot be activated in an alkaline solution during its incubation, the M.C.F.E. of pH 8.5, in which the inactive protease had been fully activated, was added to the early K.C.F.E. of pH 8.5. The protease activity in such mixture increased during its incubation at pH 8.5. If the boiled M.C.F.E. of pH 8.5 was added to the early K.C.F.E. in question, the inactive protease in it was not activated during its incubation at pH 8.5. When a carboxypeptidase, instead of M.C.F.E., was added to the early K.C.F.E the inactive protease was again activated during its incubation at pH 8.5 (Table VIII).

6. Effect of pH on the Protease Activity in the Cell Free Extract (M.C.F.E.) Obtained from Cells of Culture on M-medium.

The M.C.F.E. adjusted to various pH's were incubated with potato-inhibitor or EDTA or both substances at 30°C for 10 minutes.

And then, the protease activity on each incubation mixture was determined separately. As is shown in Fig. 3, the protease activity which remained after the

5) T. Mizunuma and I. Nobuyoshi, This Journal, **22**, 35 (1957).

TABLE VII. EFFECT OF INCUBATION pH AND PHOSPHATE IONS ON THE INCREASE OF PROTEASE ACTIVITY IN SIX KINDS OF CELL FREE EXTRACT

Kind of C.F.E.	Cultural time	pH of medium	Incubation pH	Phosphate buffer	Incubation time (mins) 0	90	180	360
K.C.F.E.	24 hrs	6.0	6.0	added	0.036	0.084	0.145	0.155
			8.5	added	0.036			0.035
			6.0	no	0.064	0.235	0.275	0.281
				added	0.051	0.225	0.268	0.248
	48 hrs	6.4	8.5	added	0.044			0.052
			6.0	added	0.073	0.195	0.252	0.248
	72 hrs	7.0	8.5	added	0.063	0.086	0.104	0.154
M. C. F. F.	24 hrs	7.0	6.0	added	0.056	0.121	0.173	0.194
			8.5	added	0.048	0.095	0.125	0.124
			6.0	no	0.117	0.205	0.281	0.353
				added	0.097	0.195	0.254	0.282
	48 hrs	7.0	8.0	added	0.095	0.174	0.235	0.292
			6.0	added	0.177	0.254	0.283	0.352
	72 hrs	7.0	8.0	added	0.151	0.225	0.295	0.307

1 g of dry mycelia obtained from three periods of culture on two kinds of media (K-medium and M-medium) was extracted with 15 ml of distilled water or with 15 ml of M/15 phosphate buffer, and each cell free extract were incubated at pH 6.0 or pH 8.5, and the protease activity in each cell free extract was determined as usual during their incubation.

TABLE VIII. EFFCT OF CARBOXYPEPTIDASE ON THE K-CELL FREE EXTRACT

		Incubation time (mins.) 0	160	320
Control (pH 8.5)		0.066	0.097	0.076
Carboxypeptidase	1	0.143	0.172	0.231
〃	0.5	0.129	0.132	0.172
〃	0.1	0.113	0.121	0.175
Zn^{++}		0.064	0.096	0.060
M. C. F. E.		0.100	0.122	0.253
Carboxypeptidase (blank)		0.044	0.041	0.047
M. C. F. E. (blank)		0.101	0.113	0.130
Control (pH 6.0)		0.066	0.198	0.227

K. C. F. E. was used same one as is shown in Fig. 3.
Original solution of carboxypeptidase being added to K. C. F. E. was used as about 100γ/ml. M. C. F. E. was used after the activation had been finished, and all mixtures were incubated at pH 8.5, at 30°C.

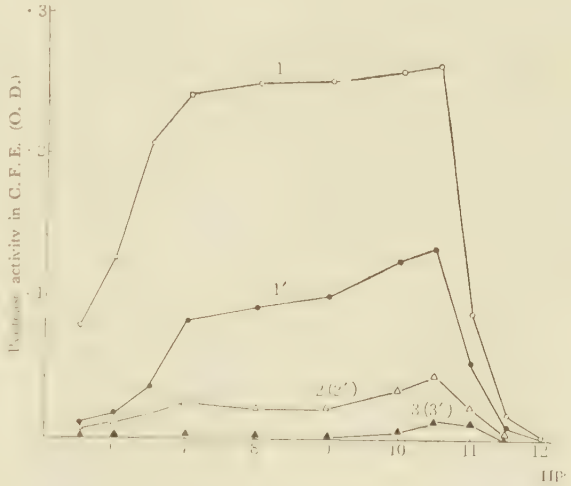

FIG. 3. Effect of pH on the Protease Activity in Cell Free Extract with and without Potato-Inhibitor and EDTA.

Curve 1, without any inhibitors; curve 2, after activation with potato-inhibitor; curve 3, after activation with potato-inhibitor and EDTA; curve 1, before activation without any inhibitors.

treatment with potato-inhibitor showed two optimal pH's, one around pH 10.5 even after inhibition with potato-inhibitor and EDTA.

DISCUSSION

From the experimental results that the in-

crease of protease activity in a cell free extract was suppressed by a dialysis of the cell free extract against EDTA at 0°C and that this suppression of activation was recovered by the addition of some metallic ions (Fig. 1), three possible explanations may be considered concerning the mechanism of activation of inactive protease. The first is a case in which the inactive protease is activated by a kinase which requires some metallic ions for its action. The second is a case in which some metallic ions are taken into proenzyme (inactive protease), making inactive protease become active. The third is a case where the activation of inactive protease goes spontaneously and some metallic ions accelerate such spontaneous activation.

Recently several investigations[6] have been reported in which some metals are taken into proenzyme, resulting in the activation of proenzyme. The experimental result that the activation of proenzyme in the cell free extract was not suppressed by dialysis of the cell free extract against distilled water shows that the activation in question is not related with the removal of free metallic ions from the cell free extract. Moreover, the activation was not inhibited by the incubation of cell free extract with EDTA at 30°C, but was inhibited by the dialysis of cell free extract against EDTA at 0°C. Therefore, the suppression effect of EDTA on the activation may be due to the removal of some strongly bound metals from a high molecular substance (protein, perhaps enzyme) in the cell free extract. It has been recently reported[6] that *Pseudomonas* protease[6], which becomes active from proenzyme by taking Ca^{++} into it, was completely inactivated by the heat treatment in the presence of EDTA, but was not inactivated by the same treatment without EDTA. On the other hand, heat treatment with EDTA or dialysis against EDTA for a long time had no effect on mold alkaline protease activity. Accordingly, it is believed that no metallic ions are involved in the action of alkaline protease. Yasui[7], using radioactive zinc, recognized that zinc ions, although having an accelerating effect on the activation of inactive protease in the cell free extract, did not move to the position of active alkaline protease in paper electrophoresis.

These results exclude a possibility that some metallic ions are taken into the proenzyme in question resulting in its activation.

Tôyama et al[4] observed that the optimum pH for the activation of proenzyme formed by *Aspergillus oryzae* W-1 lies between 5 and 6, that the activation is not accelerated by the addition of trypsin, and that the activity toward casein was not maximal at pH 5~6. From these results they negated the presence of a kinase for the activation of zymogen of *Aspergillus oryzae* W-1.

But, it is well known that[8,9] a very small amount of enzyme may be effective as a kinase for the activation of zymogen, and it is also observed that[10,11] a protease may have different pH optima for different substrates, for example, for a protein and a synthetic substrate. Moreover, it is recognized in many papers as in the case shown in Fig. 3 that *Aspergillus oryzae*[12~15] and *Aspergillus sojae*[16] produce various kinds of proteolytic enzymes which often show single pH activity curves when measured as a mixture.

Accordingly, it would be premature to conclude from Tōyama's report that there is no kinase.

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Gjessing[2] et al. recognized that the proesterase is activated spontaneously and that activation is dependent upon ionic strength and pH. Tôyama[4] et al. recognized also that the proenzyme was not activated at all at a pH above 7.4 and that phosphate ions accelerated markedly the activation of proenzyme. But, as is shown in Fig. 2, it was observed the activation of inactive protease in the cell free extract proceeds during its incubation near pH 10 as well as near pH 5. And as is shown in Table IX, the later K.C.F.F. (obtained from the mycelia grown on K-medium for 72 hours) had ability to increase protease activity during its incubation at pH 8.5. Moreover, the early K.C.F.E., which had no ability to increase protease activity at pH 8.5, increased protease activity during incubation at pH 8.5 after the addition of the M.C.F.E. (Fig. 4). When the boiled M.C.F.E. was added to the early K.C.F.E., however, the activation did not occur at pH 8.5.

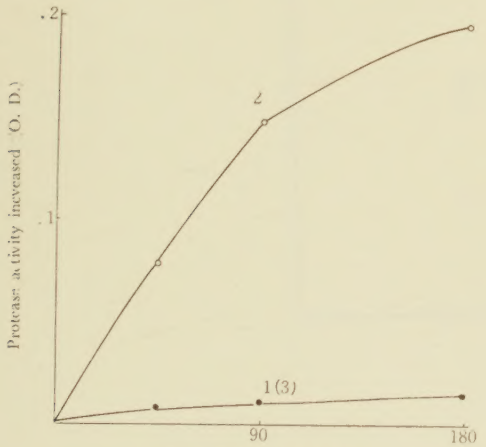

FIG. 4. Effect of Activated M. C. F. E. on the Increase in Protease Activity of K. C. F. E.

K. C. F. E. was prepared from the mycelia of *Aspergillus sojae* KS grown on K-medium at 30°C for 36 hours. M. C. F. E. was prepared from the mycelia grown on M-medium at 30°C for 48 hours, and was incubated at 30°C, at pH 6.0 for 5 hours befor the addition to K. C. F. E.. Curve 1, K. C. F. E. at pH 8.5, at 30°C; Curve 2; K. C. F. E with activated M. C. F. E. at pH 8.5, at 30°C; curve 3, K. C. F. E. with boiled activated M. C. F. E. at 30°C, at pH 8.5.

Accordingly, it was proved that the inactive protease is activated by a certain enzyme in a basic solution. And it is resonable to assume that this enzyme is formed by *Aspergillus sojae* KS in all periods of growth on M-medium and in the later period of growth on K-medium. When a carboxypeptidase obtained from pancreas was added to the K.C.F.E. the protease activity in it increased during its incubation at pH 8.5. Several kinds of metallic ions such as Pb^{++} and Zn^{++}, which are essential to carboxypeptidase action, accelerated markedly the activation of proenzyme in the cell free extract. These results show therefore that the kinase in question has perhaps a similar action as carboxypeptidase.

The inactive protease in the M.C.F.E. and in the K.C.F.E. obtained from the cultures of *Aspergillus sojae* KS was activated during their incubation at pH 6 with and without phosphate ion as is shown in Table VII, while the inactive protease obtained from the mycelia of *Aspergillus oryzae* was not activated without phosphate ions in agreement with Tôyama's results. But since the presence of a kinase which activates the inactivate protease at around pH 5 was not established, there remained a possibility that the inactive protease may be activated spontaneously during its incubation at an acid pH. These possibilities will be further investigated.

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